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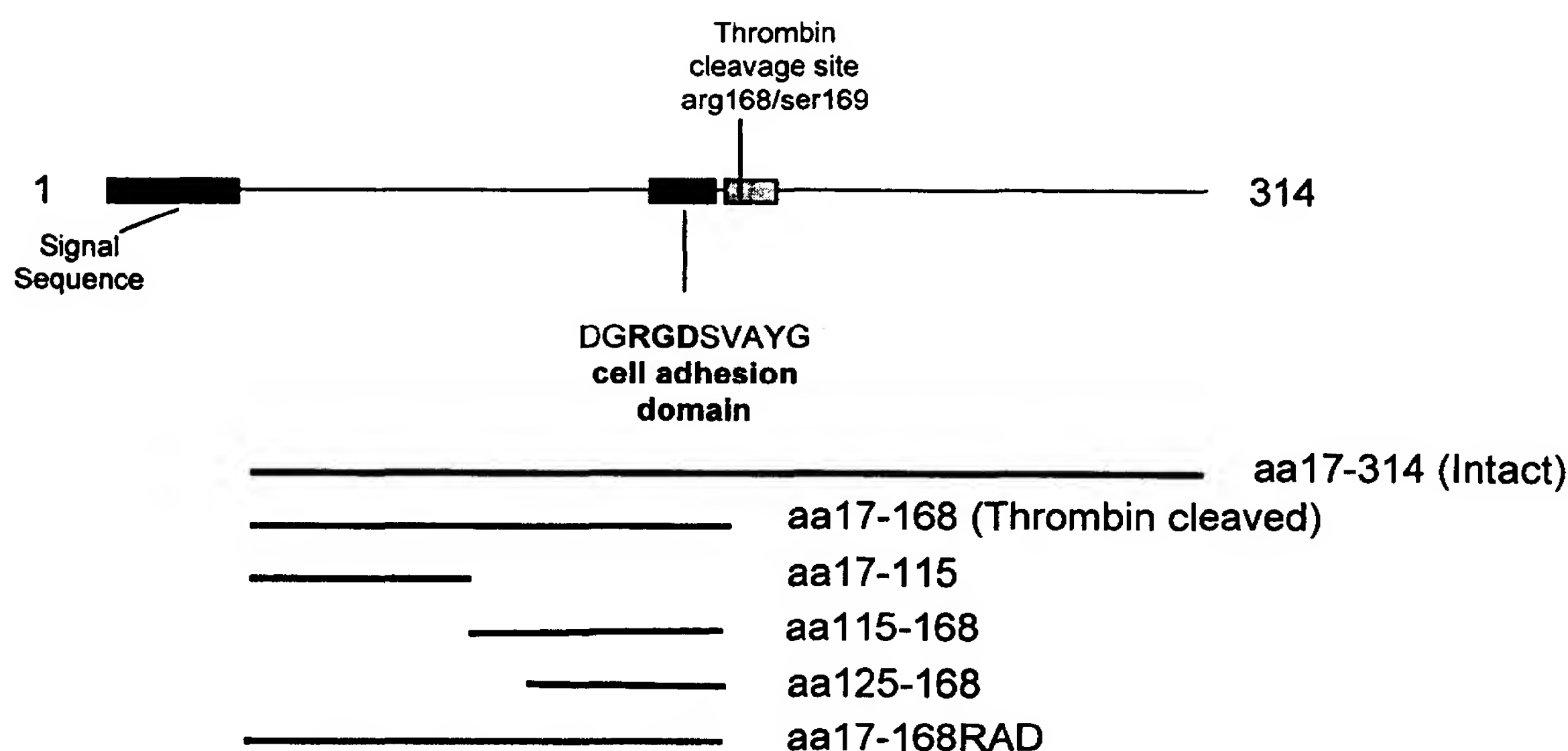
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(54) Title: METHOD OF SCREENING FOR INHIBITORS OF OSTEOPONTIN



(57) Abstract: Amino acids (136 to 142) and amino acids (162 to 168) of human osteopontin constitute two sites at which osteopontin interacts with $\alpha 4$ integrins. Products capable of disrupting those interactions are useful in therapy, particularly in the treatment of inflammatory diseases.



WO 01/71358 A1

METHOD OF SCREENING FOR INHIBITORS OF OSTEOPONTIN

Field of the invention

The invention relates to medical products and, in particular, products for use
5 in the treatment of diseases associated with inflammation. The invention also relates
to methods for identifying products which are useful in therapy, particularly in the
treatment of diseases associated with inflammation.

Background to the invention

10 The integrins comprise a large family of heterodimeric transmembrane
receptors that mediate both cell-cell and cell-matrix interactions. They engage
numerous ligands and regulate a variety of cellular and physiological processes such
as cell proliferation, apoptosis, migration, differentiation, inflammation and tissue
remodelling. The $\alpha 4 \beta 1$ integrin is expressed mainly on leukocytes but is also found
15 on smooth muscle cells and tumour cells. It has received particular attention because
of its putative role in modulating the inflammatory response, promoting the
exfiltration of leukocytes from the circulation. The $\alpha 4 \beta 7$ integrin is also thought to
play a role in the inflammatory process.

Osteopontin (OPN) is an RGD containing extracellular matrix protein
20 expressed by a number of cell types including osteoclasts, osteoblasts, macrophages,
activated T-cells, smooth muscle cells and epithelial cells. It is present in several
tissues including bone, kidney, placenta, smooth muscle and secretory epithelia and
is associated with normal tissue remodelling processes such as bone resorption,
angiogenesis, wound healing and tissue injury as well as certain diseases including
25 restenosis, atherosclerosis, renal diseases and tumorigenesis. Upon infection and
damage OPN expression is rapidly upregulated by T cells and macrophages. It acts
as a chemoattractant for smooth muscle cells, and may facilitate further recruitment
and activation of both T-cells and macrophages. In addition it may also act as a
costimulatory molecule for T-cells. The diverse functions of osteopontin suggest that
30 it may be important in both the immune-response and tissue remodelling.

OPN exerts many of its biological effects by interacting with integrins. It constitutively binds $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 1$ and $\alpha 8\beta 1$ via a central RGD motif, while proteolytic modification of OPN by thrombin cleavage at aa168 reveals cryptic binding sites for $\alpha 9\beta 1$ and $\alpha 5\beta 1$.

5

Summary of the invention

This invention is based on the finding that $\alpha 4$ integrins bind the extracellular matrix protein osteopontin (OPN) and on the identification of two novel peptide sequences within OPN which bind the $\alpha 4$ integrins $\alpha 4\beta 1$ and $\alpha 4\beta 7$. The importance of an interaction between OPN and $\alpha 4$ integrins is that it provides a link between these proteins, immuno-modulation and inflammatory diseases. Thus the interaction between OPN and $\alpha 4$ integrins, such as $\alpha 4\beta 1$ and $\alpha 4\beta 7$, is an important target for the identification of products which may be used as immuno-modulators or used in the treatment of inflammatory disease.

In order to bind an $\alpha 4$ integrin, the second motif typically needs a free acidic carboxyl terminus. Proteolytic cleavage of OPN at amino acid 168 probably generates this *in vivo*. In the present invention embodiments using polypeptides with the second motif or functional variants of it will typically have either the free carboxylic acid terminus already present or will be able to be cleaved, preferably proteolytically, to give rise to such an acidic group.

20

According to the present invention there is thus provided a method for a method for identifying a product which is capable of disrupting an interaction between amino acids 136 to 142 and/or amino acids 162 to 168 of human osteopontin (OPN) and an $\alpha 4$ integrin, which method comprises:

25

- (i) providing, as a first component, a polypeptide which is (a) up to 50 amino acids in length and which comprises the amino acid sequence $X_1X_2\text{FPTDLP}X_3X_4$ or a functional variant thereof and/or the amino acid sequence $X_7X_6X_5\text{SVVYGLR}$ or a functional variant thereof or (b) a fusion protein wherein the polypeptide (a) is fused to a carrier

polypeptide, wherein:

X_1 is absent or any amino acid and, if X_1 is present, X_2 is absent or any amino acid;

X_3 is absent or any amino acid and, if X_3 is present, X_4 is absent or any amino acid; and

X_5 is absent or any amino acid and, if X_5 is present, X_6 is absent or any amino acid and, if X_6 is present, X_7 is absent or any amino acid;

(ii) providing, as a second component, an $\alpha 4$ integrin or a functional variant thereof;

(iii) contacting the two components with a test product under conditions that would permit the two components to interact in the absence of the test product; and

(iv) determining whether the test product is capable of disrupting the interaction between the first and second components.

Typically the $\alpha 4$ integrin used as a second component is $\alpha 4\beta 1$.

The invention also provides:

a method for identifying a product which is capable of binding to amino acids 136 to 142 and/or amino acids 162 to 168 of human OPN, which method comprises:

(i) providing, as a first component, a polypeptide which is (a) up to 50 amino acids in length and which comprises the amino acid sequence X_1X_2 FPTDLP X_3X_4 or a functional variant thereof and/or the amino acid sequence $X_7X_6X_5$ SVVYGLR or a functional variant thereof or (b) a fusion protein wherein the polypeptide (a) is fused to a carrier polypeptide, wherein:

X_1 is absent or any amino acid and, if X_1 is present, X_2 is absent or any amino acid;

X_3 is absent or any amino acid and, if X_3 is present, X_4 is absent or any amino acid; and

X_5 is absent or any amino acid and if X_5 is present, X_6 is absent or any

amino acid and, if X_6 is present, X_7 is absent or any amino acid;

- (ii) contacting the first component with a test product under conditions that, in the presence of an $\alpha 4$ integrin but the absence of the test product, would lead to an interaction between the first component and an $\alpha 4$ integrin; and

- (iii) determining whether the test product is capable of binding to the first component;

a test kit suitable for use in identifying a product which is capable of disrupting an interaction between amino acids 136 to 142 and/or amino acids 162 to 168 of human OPN an $\alpha 4$ integrin, which kit comprises:

- (a) a first component as defined above; and
(b) an $\alpha 4$ integrin or a functional variant thereof;

a test kit suitable for identifying a product which is capable of binding to amino acids 136 to 142 and/or amino acids 162 to 168 of human OPN, which kit comprises:

- (a) a first component as defined above; and
(b) means for identifying whether a test product binds to the said first component.

a product identified by a method of the invention ("a product of the invention");

a product of the invention for use in a method of treatment of the human or animal body by therapy;

use of a product of the invention in the manufacture of a medicament for use in immuno-modulation or the treatment of an inflammatory disease, a granulomatous disease, fibrotic disease, multiple sclerosis or a bacterial or viral infection;

a pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent and, as active ingredient, a product of the invention;

a method for treating a host requiring immuno-modulation or suffering from an inflammatory disease, a granulomatous disease, fibrotic disease, multiple

sclerosis or a bacterial or viral infection which method comprises administering to the host an effective amount of a product of the invention; a polypeptide (a) or (b) as defined in relation to a method of the invention, excluding the polypeptide SVVYGLR;

5 an antibody capable of binding a polypeptide (a) or (b) ("an antibody of the invention");

a polypeptide (a) or (b) or an antibody of the invention; for use in a method of treatment of the human or animal body by therapy;

use of a polypeptide (a) or (b) or an antibody of the invention, in the

10 manufacture of a medicament for use in immuno-modulation or the treatment of an inflammatory disease, a granulomatous disease, fibrotic disease, multiple sclerosis or a bacterial or viral infection;

a pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent and, as active ingredient, a polypeptide (a) or (b) as defined

15 in a method of the invention, or an antibody of the invention; and

a method for treating a host requiring immuno-modulation or suffering from an inflammatory disease, a granulomatous disease, fibrotic disease, multiple sclerosis or a bacterial or viral infection, which method comprises administering to the host an effective amount of a polypeptide (a) or (b) or an

20 antibody of the invention;

use of a polypeptide (a) or (b) in a method for identifying a product which is capable of disrupting an interaction between amino acids 136 to 142 and/or amino acids 162 to 168 of human OPN and an $\alpha 4$ integrin;

use of a polypeptide (a) or (b) in a method for identifying a product which is

25 capable of binding to amino acids 136 to 142 and/or amino acids 162 to 168 of human OPN; and

a method of diagnosis of an inflammatory disease, a granulomatous disease, fibrotic disease, multiple sclerosis or a bacterial or viral infection which method comprises contacting an antibody of the invention with a biological

30 sample from a human or animal subject and determining whether the

antibody binds to amino acids 136 to 142 and/or amino acids 162 to 168 of OPN, thereby to determine the disease state of the subject.

Brief description of the figures

5 Figure 1 shows a schematic diagram of the recombinant OPN GST-fusion proteins used for adhesion assays. All OPN fusion proteins were expressed in the lon protease deficient bacterial strain UT5600. Each protein preparation was over 95% pure as judged by mass spectrometry and SDS-PAGE analysis and not subject to protease digestion. The fusion protein aa17-314 represents full length OPN. The
10 fusion protein aa17-168 represents the thrombin cleaved fragment, mimicking proteolytic cleavage with thrombin at amino acid residue 168. The fusion protein aa17-115 represents a further deletion from the C-terminus of OPN past the RGD motif. The two fusion proteins aa115-168 and aa125-168 represent deletions from the N-terminus of the thrombin cleaved fragment. The fusion protein aa17-168RAD
15 represents the thrombin cleaved fragment of OPN in which the RGD motif has been mutated to RAD.

 Figure 2 shows that J6 and HL60 cells adhere to OPN via $\alpha 4\beta 1$. (A) J6 cells were allowed to adhere to wells coated with a GST fusion protein representing intact osteopontin (aa17-314) at 20 μ g/ml. Cells were incubated for 35 minutes at 37°C in
20 the presence of or absence of various function blocking anti-integrin antibodies at 10 μ g/ml. To prebind the antibodies cells were preincubated on ice for 10 minutes before being moved to 37°C. Each data point represents the mean \pm SD of duplicate points. (B) HL60 cells were allowed to adhere to wells coated with a GST fusion protein representing intact osteopontin (aa17-314) at 20 μ g/ml. To stimulate $\alpha 4\beta 1$
25 binding to OPN, 50ng/ml TPA was also included. Cells were incubated for 35 minutes at 37°C in the presence or absence of various function blocking anti-integrin antibodies at 10 μ g/ml. To prebind the antibodies cells were preincubated on ice for 10 minutes before being moved to 37°C. Each data point represents the mean \pm SD of duplicate points.

Figure 3 shows that $\alpha 4\beta 1$ binds within the N-terminal fragment of OPN generated by thrombin cleavage. J6 cells were allowed to attach to wells coated with various concentrations of GST-fusion proteins of intact OPN (aa17-314), thrombin cleaved OPN (aa17-168) and a OPN fragment truncated past the RGD motif (aa17-115) as well as GST fusions of the CS-1 region of fibronectin (CS-1), and repeats 8-10 of fibronectin (FN8-10). Cells were incubated for 35 minutes at 37°C in the presence (closed circles) or absence (open circles) of the small molecule inhibitor of $\alpha 4\beta 1$, BIO1211, at 100nM. Each data point represents the mean \pm SD of duplicate points.

Figure 4 shows that $\alpha 4\beta 1$ binds a site within aa125-168 distinct from the RGD motif. (A) J6 cells were allowed to attach to wells coated with various concentrations of GST-fusion proteins of OPN aa115-168 and aa125-168 which represent two fragments of thrombin cleaved OPN differentially truncated from the N-terminus. Cells were incubated for 35 minutes at 37°C in the presence (closed circles) or absence (open circles) of the small molecule inhibitor of $\alpha 4\beta 1$, BIO1211, at 100nM. Each data point represents the mean \pm SD of duplicate points. (B) J6 cells were allowed to attach to wells coated with various concentrations of a GST-fusion protein of thrombin cleaved OPN in which the RGD motif is mutated to RAD (aa17-168RAD). Cells were incubated for 35 minutes at 37°C in the presence (closed circles) or absence (open circles) of the small molecule inhibitor of $\alpha 4\beta 1$, BIO1211, at 100nM. Each data point represents the mean \pm SD of duplicate points.

Figure 5 shows the definition of $\alpha 4\beta 1$ interacting peptides within OPN. (A) The region containing the $\alpha 4\beta 1$ binding site, aa125-168, was divided into 5 overlapping peptides which were expressed as GST fusion proteins. The two peptides which supported $\alpha 4\beta 1$ dependent adhesion are shown in bold. (B) J6 cells were allowed to attach to overlapping GST fusion proteins spanning aa125-168 as shown in (A). All fusion proteins were coated at 20 μ g/ml and J6 cells were incubated at 37°C for 35 minutes. Each data point represents the mean \pm SD of duplicate points. (C) J6 cells were allowed to attach to wells coated with GST-fusion

proteins of OPN aa132-146 and aa153-168, as well as GST-CS-1. Cells were incubated for 35 minutes at 37°C in the presence (dark grey) or absence (light grey) of 100nM of the small molecule inhibitor of $\alpha 1$, BIO1211. Each data point represents the mean \pm SD of duplicate points.

5 Figure 6 shows an alignment of the $\alpha 4\beta 1$ binding region, aa125-168. The human, bovine, pig, rabbit, rat and mouse sequences incorporating the $\alpha 4\beta 1$ interacting peptides, aa125-168 are shown. The region identified within the human protein as $\alpha 4\beta 1$ interacting peptides are underlined.

10 Figure 7 shows the degree of inhibition of the $\alpha 4\beta 1$ -CS-1 interaction by various OPN derived peptides. J6 cells were allowed to attach to wells coated with GST-CS-1 in the presence of CS-1 peptide (open circles) and scrambled control peptide (closed circles) shown in (A); SVVYGLR (open circles) and the scrambled control peptide, GRVLYSV (closed circles) shown in (B); FPTDLPA (open circles) and the scrambled control peptide, PDFPLAT (closed circles) shown in (C). Cells were incubated at 37°C
15 for 35 minutes. This data is representative of at least four similar experiments and each data point represents the mean \pm SD of duplicate points.

20 Figure 8 shows that $\alpha 4\beta 7$ binds the SVVYGLR motif of osteopontin. RPMI8866 cells were allowed to adhere to a GST fusion protein incorporating amino acid residues 153-168 of osteopontin SVVYGLR peptide in the presence or absence of anti-functional integrin antibodies (20 μ g/ml), as indicated. Cells were incubated at 37°C for 20 minutes. This data is representative of at least four similar experiments and each data point represents the mean \pm SD of duplicate points.

25 Figure 9 shows that RPMI8866 cells bind zz-MAdCAM via $\alpha 4\beta 7$. RPMI8866 cells were allowed to adhere to zz-MAdCAM in the presence or absence of anti-functional integrin antibodies (20 μ g/ml), as indicated. Cells were incubated at 37°C for 20 minutes. This data is representative of at least four similar experiments and each data point represents the mean \pm SD of duplicate points.

Figure 10 shows that peptide SVVYGLR inhibits $\alpha 4\beta 7$ binding to MAdCAM. RPMI8866 cells were adhered to zz-MAdCAM in the presence of varying

concentrations of parent SVVYGLR or scrambled GRVLYSV peptides. This data is representative of at least three experiments, and each data point represents the mean \pm SD of duplicate points.

Figure 11 shows that the acidic carboxy terminal COOH group (Arg 168) of the SVVYGLR motif is required for inhibition of $\alpha 4\beta 1$ and $\alpha 4\beta 7$. (A) J6 cells were adhered to GST-CS-1 in the presence of varying concentrations of parent SVVYGLR-COOH, capped SVVYGLR-CONH₂ or scrambled GRVLYSV-COOH peptides. This data is representative of at least three experiments. (B) RPMI8866 cells were adhered to zz-MAdCAM in the presence of varying concentrations of parent SVVYGLR, capped SVVYGLR-CONH₂ or scrambled GRVLYSV peptides. This data is representative of at least three experiments, and each data point represents the mean \pm SD of duplicate points.

Figure 12 shows the identification of key residues within SVVYGLR required for inhibition of $\alpha 4\beta 1$ and $\alpha 4\beta 7$ binding. J6 and RPMI8866 cells were allowed to adhere to CS-1 and zz-MAdCAM in the presence of a series of SVVYGLR peptides in which each residue was individually mutated to alanine. Each residue mutated to alanine is indicated. The data are expressed as a relative potency (RP) compared to the parent peptide SVVYGLR, and represent to mean of 4 experiments.

Figure 13 shows a summary of the key elements of the SVVYGLR motif. Leu167 and the free carboxy terminal -COOH group of Arg168 are required for the interaction with $\alpha 4\beta 1$ and $\alpha 4\beta 7$. Val163, Val164 and Tyr165 make a minor contribution to binding. Mutating Arg168 and Gly166 introduces a degree of selectivity between $\alpha 4\beta 1$ and $\alpha 4\beta 7$.

Description of Sequence Listing

SEQ ID NO: 1 shows the amino acid sequence of human osteopontin (GenBank Accession Number: X13694).

Detailed description of the invention

We have shown that two new peptide motifs in OPN determine its ability to interact with $\alpha 4$ integrins. The interaction between osteopontin (OPN) and $\alpha 4$ integrins appears to be important in, for example, immuno-modulation, in inflammatory disease and in granulomatous disease and in fibrotic disease and multiple sclerosis. Thus, disruption of the interaction between the new OPN motifs and $\alpha 4$ integrins offers a target for therapy by immuno-modulation and therapy of various types of diseases. One of the motifs comprises amino acids 136 to 142 of OPN and the other comprises amino acids 162 to 168 of OPN.

The motif comprising amino acids 162 to 168 of OPN has a higher affinity for $\alpha 4$ integrins than the motif comprising amino acids 136 to 142 of OPN. The motif comprising amino acids 162 to 168 has its highest affinity for OPN when it has a free carboxyl group (carboxylic acid) at amino acid residue 168. It is likely that *in vivo* such a free carboxyl group will be present after proteolytic cleavage of OPN at amino acid 168. In embodiments of the invention which use polypeptides carrying the second motif, or a functional variant thereof, the polypeptide will either have such an acidic carboxyl group or will be able to be cleaved to give rise to such a group.

The invention provides methods for identifying a product capable of disrupting an interaction between amino acids 136 to 142 and/or amino acids 162 to 168 of OPN, typically human OPN, and an $\alpha 4$ integrin.

A two component system is used. The two components are contacted in the presence of a test product. The contacting step is typically carried out under conditions that would permit the interaction of the two components being tested in the absence of the test product. The degree of interaction between the two components may be determined so that the degree of disruption of the interaction by the test substance can be determined.

The first component can be a polypeptide (a) which is typically up to about 50 amino acids in length and which comprises the amino acid sequence

X₂X₁FPTDLPAX₃X₄ or a functional variant thereof and/or the amino acid sequence

$X_7X_6X_5SVVYGLR$ or a functional variant thereof, wherein:

- X_1 may be absent or any amino acid and, if present, it is preferably D. If X_1 is present, X_2 may be absent or any amino acid and, if X_2 is present, it is preferably T. Preferably, X_1 and X_2 are both present and X_1 is D and X_2 is T.
- 5 - X_3 may be absent or any amino acid and, if present, it is preferably T. If X_3 is present, X_4 may be absent or any amino acid and, if X_4 is present, it is preferably E. Preferably X_3 and X_4 are both present and X_3 is T and X_4 is E.
- X_5 may be absent or any amino acid and, if present, it is preferably D. If X_5 is present, X_6 may be absent or any amino acid and, if X_6 is present, it is preferably G or A. If X_6 is present, X_7 may be absent or any amino acid and, if X_7 is present, it is preferably R. Preferably, X_5 , X_6 and X_7 are all present and X_5 is D, X_6 is G or A and X_7 is R.

Thus, the polypeptide (a) is typically a polypeptide which is up to about 50 amino acids in length and comprises the sequence FPTDLPA, DFPTDLPA, TDFPTDLPA, FPTDLPAT, FPTDLPATE, DFPTDLPAT, DFPTDLPATE, TDFPTDLPAT or TDFPTDLPATE or functional variants of any thereof and/or SVVYGLR, DSVVYGLR, GDSVVYGLR, RGDVVYGLR or RADVVYGLR or functional variants of any thereof. Alternatively, the polypeptide (a) may consist essentially of an above mentioned amino acid sequence or a functional variant thereof. The polypeptide (a) can comprise or consist essentially of both $X_2X_1FPTDLPAX_3X_4$ and $X_7X_6X_5SVVYGLR$ or functional variants thereof.

A polypeptide (a) may comprise a functional variant of $X_2X_1FPTDLPAX_3X_4$ and/or $X_7X_6X_5SVVYGLR$. A functional variant is a sequence which is similar to $X_2X_1FPTDLPAX_3X_4$ or $X_7X_6X_5SVVYGLR$ and which retains $\alpha 4$ integrin binding activity, typically it will retain $\alpha 4\beta 1$ binding activity. Typically, the binding activity of a functional variant may be substantially the same as the binding activity of $X_2X_1FPTDLPAX_3X_4$ or $X_7X_6X_5SVVYGLR$. Alternatively, the binding activity of a functional variant may be greater or less than that of one of $X_2X_1FPTDLPAX_3X_4$ or $X_7X_6X_5SVVYGLR$. A functional variant typically comprises a sequence substantially similar to $X_2X_1FPTDLPAX_3X_4$ or $X_7X_6X_5SVVYGLR$.

A functional variant may have increased or decreased affinity for a particular $\alpha 4$ integrin relative to the wild type OPN motif. For example, the variant may have an increased affinity for $\alpha 4\beta 1$ or $\alpha 4\beta 7$ integrin.

The functional variant may have increased affinity for one $\alpha 4$ integrin but
5 have decreased affinity for a second $\alpha 4$ integrin. For example, the variant may have increased affinity for $\alpha 4\beta 1$ but have decreased affinity for $\alpha 4\beta 7$ or *vice versa*. Such variants may be used as therapeutic molecules to selectively act against a particular $\alpha 4$ integrin without affecting the other $\alpha 4$ integrin. Typically these variants will have amino acid changes in, or around, the binding motif or in a region affecting its
10 activity. In particular such variants may have different amino acids at the amino acid positions corresponding to amino acid residues 166 and/or 168 of the wild type OPN binding site.

Thus a functional variant will generally have at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98 or at least 99% sequence identity to
15 $X_2X_1FPTDLPAX_3X_4$ or $X_7X_6X_5SVVYGLR$, calculated over the full length of those sequences. The UWGCG Package provides the BESTFIT program which can be used to calculate identity (for example used on its default settings) (Devereux *et al* (1984) *Nucleic Acids Research* 12, p387-395). The PILEUP and BLAST algorithms can be used to calculate identity or line up sequences (typically on their default
20 settings), for example as described in Altschul S. F. (1993) *J Mol Evol* 36:290-300; Altschul, S, F *et al* (1990) *J Mol Biol* 215:403-10. Software for performing BLAST analyses is publicly available through the National Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>).

A functional variant may be a naturally occurring sequence, for example an
25 allelic variant. An allelic variant will generally be of human or non-human mammal, for example bovine or porcine, origin.

Alternatively, a functional variant may be a non-naturally occurring sequence. A non-naturally occurring functional variant may be a modified version of $X_2X_1FPTDLPAX_3X_4$ or $X_7X_6X_5SVVYGLR$ obtained by, for example, amino acid
30 substitution or deletion. 1, 2, 3, 4 or 5 amino acid substitutions or deletions may be

made. Typically, the substitutions will be conservative substitutions, for example according to the following Table. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other. Deletions are preferably deletions of amino acids from one or both ends of the sequences given in SEQ ID NOS: 1 and 2.

ALIPHATIC	Non-polar	G A P
		I L V
	Polar-uncharged	C S T M
		N Q
	Polar-charged	D E
		K R
AROMATIC		H F W Y

Functional variants may be sequences intermediate between human and non-human alleles. For example, the bovine version of the human $X_2X_1FPTDLPAX_3X_4$, motif is $X_2X_1FPTDIPTX_3X_4$, and a functional variant may contain one or both differences between the human and bovine sequence. Thus, a functional variant may be $X_2X_1FPTDX_aPX_bX_3X_4$, wherein X_a is L or I and X_b is A or T. Similarly, the bovine version of the human motif $X_7X_6X_5SVVYGLR$ is $X_7X_6X_5SVAYGLK$ and thus, a functional variant may be $X_7X_6X_5SVX_cYGLX_d$, wherein X_c is V or A and X_d is R or K.

A polypeptide (a) is typically up a longer polypeptide of to about 50 amino acids in length. Thus, a polypeptide (a) may have the sequence:

- (1) $(X_{aa})_nX_2X_1FPTDLPAX_3X_4, (X_{aa})_m$ or a functional variant thereof, wherein X_1 to X_4 are present, n is 0 to 39 such as 5 to 25, m is 0 to 39 such as 5 to 25, (m+n) is 0 to 39 and any X_{aa} is independently any amino acid; or

(2) $(X_{aa})_p X_7 X_6 X_5 SVVYGLR$ or a functional variant thereof, wherein X_5 to X_7 are present, p is 0 to 40 such as 5 to 25 and X_{aa} is independently any amino acid.

Put another way, the motif FPTDLPA or a functional variant thereof may be extended by the addition of from 1 to about 43 amino acids, for example from 5 to 25 amino acids, and the motif SVVYGLR may be extended by the addition of from 1 to about 43 amino acids, for example from 5 to 25 amino acids.

The OPN motif comprising amino acids 136 to 142 of OPN, or a functional variant of this motif, may be situated at any point of a polypeptide which is up to about 50 amino acids in length. For example, this OPN motif may be situated at the C- or N- terminal end of a polypeptide. Generally, however, the OPN motif will be situated substantially in the centre of a polypeptide, i.e. m and n are typically a similar integer.

The binding motif comprising amino acids 162 to 168 of OPN, or a functional variant thereof, will typically be present at the C-terminus of the polypeptide (a). The polypeptide (a) may have amino acid residues C terminal to the motif, however if this is the case these will typically be removed at some stage by proteolytic cleavage prior to binding to the $\alpha 4$ integrin.

The flanking sequences (i.e. those represented by X_{aa} in the formulae above) may be of any suitable sequence. However, the flanking sequences should be such that interaction between the OPN motif and the $\alpha 4$ integrin can occur. Thus, the flanking sequences allow the OPN motif to adopt an appropriate conformation for binding to the $\alpha 4$ integrin and will be capable of presenting the motif. It can be easily determined whether the motif is presented by determining whether the polypeptide comprising the motif is capable of binding to an $\alpha 4$ integrin. In addition, the flanking sequences typically should substantially not interact with the $\alpha 4$ integrin.

Preferably, the flanking sequences are the same or substantially similar to the sequences that flank the OPN motifs in the wild type human OPN polypeptide or are functional variants thereof. For example, a polypeptide (a) may comprise the sequence defined by amino acids 132 to 142, 136 to 146 or 132 to 146 and/or 156 to

148 of SEQ ID NO: 1 or a functional derivative thereof. Indeed, polypeptides that comprise human OPN flanking sequences are preferred as they may provide for increased binding to an $\alpha 4$ integrin.

Therefore:

5 (X_{aa})_m may be V, LV, ELV, DELV, SDELV, ESDELV, DESDELV, SDESDELV, HSDESDELV or HHSDESDELV etc. or functional variants thereof, for example;

(X_{aa})_n may be V, VF, VFT, VFTP, VFTPV, VFTPVV, VFTPVVP, VFTPVVPT, VFTPVVPTV or VFTPVVPTVD etc. or functional variants thereof, 10 for example;

(X_{aa})_p may be D, YD, TYD, DTYD, VDTYD, TVDTYD, PTVDTYD, VPTVDTYD, VVPTVDTYD, or PVVPTVDTYD etc. or functional variants thereof, for example; and

(X_{aa})_q may be S, SK, SKS, SKSK, SKSKK, SKSKKF, SKSKKFR, 15 SKSKKFRR, SKSKKFRRP or SKSKKFRRPD etc. or functional variants thereof, for example.

Preferred polypeptides (a) comprise both OPN motifs. The two motifs may be placed adjacent to each other with no intervening sequences. However, typically a sequence will intervene between the two motifs. A preferred intervening sequence is 20 one which is substantially similar to the sequence of amino acids 143 to 157 of the human OPN sequence or a functional variant thereof. Polypeptides which comprise both motifs may be useful if high affinity binding to an $\alpha 4$ integrin requires both OPN motifs.

Polypeptides (b) for use in the invention comprise a polypeptide (a) fused to a 25 carrier polypeptide. Additional amino acid residues may be provided at one or both termini of a polypeptide (a) for the purpose of providing a carrier polypeptide, by which the polypeptide can be, for example, affixed to a label, solid matrix or carrier. Thus the polypeptide (b) may be in the form of a fusion polypeptide which comprises heterologous sequences. Indeed, in practice it may often be convenient to use fusion 30 polypeptides. This is because fusion polypeptides may be easily and cheaply

produced in recombinant cell lines, for example recombinant bacterial or insect cell lines. Typically fusion polypeptides will comprise a polypeptide sequence as described above and a carrier or linker sequence. The carrier or linker sequence will typically be derived from a non-human, preferably a non-mammalian source, for example a bacterial source. This is to minimize the occurrence of non-specific interactions between sequences in the fusion polypeptide and the $\alpha 4$ integrin.

In the case of fusion proteins comprising the SVVYGLR motif, or a functional variant thereof, amino acids C-terminal to the arginine residue will typically be removed by proteolytic cleavage prior to binding to an $\alpha 4$ integrin.

Polypeptides may be modified by, for example, addition of histidine residues, a T7 tag or glutathione S-transferase, to assist their purification. Alternatively, the carrier polypeptide may promote secretion of the polypeptide from a cell or target expression of the polypeptide to a particular subcellular compartment. Amino acids carriers can be from 1 to 400 amino acids in length or more typically from 5 to 200 residues in length. The polypeptide may be linked to a carrier polypeptide directly or via an intervening linker sequence. Typical amino acid residues used for linking are tyrosine, cysteine, lysine, glutamic acid or aspartic acid.

Suitable polypeptides for use as a first component may be chemically modified, for example, post translationally modified. For example they may be glycosylated or comprise modified amino acid residues. Polypeptides can be in a variety of forms of polypeptide derivatives, including amides and conjugates with polypeptides.

Chemically modified polypeptides also include those having one or more residues chemically derivatized by reaction of a functional side group. Such derivatized side groups include those which have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups and formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-

benzylhistidine.

Also included as chemically modified polypeptides are those polypeptides which contain one or more naturally occurring amino acids derivatives of the twenty standard amino acids. For example, 4-hydroxyproline may be substituted for proline
5 or homoserine may be substituted for serine.

Polypeptides may carry a revealing label. Suitable labels include radioisotopes such as ^{32}P or ^{35}S , fluorescent labels, enzyme labels, or other protein labels such as biotin.

Polypeptides may be expressed using recombinant DNA techniques. For
10 example, suitable polypeptides may be expressed in, for example, bacterial or insect cell lines.

Alternatively, polypeptides may be chemically synthesized. The upper limit for a chemically synthesized polypeptide is typically about 50 amino acids. Synthetic techniques, such as a solid-phase Merrifield-type synthesis, may be
15 preferred for reasons of purity, antigenic specificity, freedom from unwanted side products and ease of production. Suitable techniques for solid-phase peptide synthesis are well known to those skilled in the art (see for example, Merrifield *et al.*, 1969, *Adv. Enzymol* 32, 221-96 and Fields *et al.*, 1990, *Int. J. Peptide Protein Res*, 35, 161-214). In general, solid-phase synthesis methods comprise the sequential
20 addition of one or more amino acid residues or suitably protected amino acid residues to a growing peptide chain.

Polypeptides for use as a first component in a method of the invention may be linear or cyclic. A linear polypeptide may be cyclised according to any suitable method (see for example Zimmer *et al.*, 1992, *Peptides*, pp.393-394, ESCOM
25 Science Publishers, BV., 1993 and Gurrath *et al.*, 1992, *Eur. J. Biochem.*, 210, 911-921). Typically, tertbutoxycarbonyl protected polypeptide methyl ester is dissolved in methanol and sodium hydroxide are added and the admixture is reacted at 20°C to hydrolytically remove the methyl ester protecting group. After evaporating the solvent, the tertbutoxycarbonyl protecting group is extracted with ethyl acetate from
30 acidified aqueous solvent. The tertbutoxycarbonyl protecting group is then removed

under mildly acidic conditions in dioxane cosolvent. The unprotected linear peptide with free amino acid and carboxy termini so obtained is converted to its corresponding cyclic polypeptide by reacting a dilute solution of the linear polypeptide in a mixture of dimethylformamide with dicyclohexylcarbodiimide in the presence of 1-hydroxy benzotriazole and N-methylmorpholine. The resultant cyclic polypeptide is purified by chromatography.

The second component comprises an $\alpha 4$ integrin or a functional variant thereof. The $\alpha 4$ integrin family comprises $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrins and either of these, or a function variant thereof, may be used in the invention. Typically $\alpha 4\beta 1$ may be used.

A functional variant of an $\alpha 4$ integrin is a polypeptide which shows $\alpha 4$ integrin-like activity in respect of the ability of $\alpha 4$ integrins to bind OPN. More specifically, the functional variant is able to bind at least one of the amino acid motifs represented by 136 to 142 and amino acids 162 to 168 of OPN. Thus, the second component may comprise, for example, a fragment of an $\alpha 4$ integrin which binds amino acids 136 to 142 and/or amino acids 162 to 168 of OPN, or a polypeptide which comprises wild type sequences for OPN binding sites and elsewhere comprises non-wild type sequences. Preferably the variant will be able to bind the amino acid motif represented by amino acids 162 to 168 of OPN.

Also, suitable functional variants may comprise non-wild type OPN binding sites, but will still be capable of binding at least one of the amino acid motifs represented by 136 to 142 and amino acids 162 to 168 of OPN and preferably the amino acid motif represented by amino acids 162 to 168 of OPN. It may be preferable to use non-wild type binding sites which show an increased binding affinity for those motifs, as compared with the binding affinity of wild type binding sites. Use of such non-wild type binding sites may allow the identification of products which are strong disrupters of OPN / $\alpha 4$ integrin interactions. Non-wild type OPN binding sequences will typically arise through substitution or deletion for example, as described for the first component above.

Second component polypeptides may be produced according to similar methods as described for first component polypeptides.

A two component assay can be carried out according to any suitable protocol, but preferably the assay is adapted so that it can be carried out in a single reaction
5 vessel and more preferably can be carried out in a single well of a plastics microtitre plate and thus can be adapted for high through-put screening. Typically, a cell adhesion assay is carried out.

In a cell adhesion assay, the first component polypeptide is coated on the walls of a suitable vessel, in particular the well of a plastics microtitre plate. In one
10 suitable assay format, the second component, produced, for example, chemically or recombinantly is simply added to the assay vessel. Binding of the second component to the first component can be followed by the use of a second component which carries a label, for example a radioactive label or a fluorescent label.

Alternatively, in another suitable assay format, cells expressing the second
15 component are added to the vessel and allowed to interact with the first component in the presence of a test product. The number of cells which bind to the first component polypeptide is then determined. This may be carried out by, for example, staining the cells and then carrying out spectrophotometry. It may be necessary to add further components to the reaction mixture in order to promote the $\alpha 4$ integrin to a suitable
20 activation state for binding to OPN. In addition, suitable control experiments may be carried out. The cell adhesion assay may be run without the test product present. In order to distinguish between non-specific interactions between the first component and cells expressing the second component, antibodies specific to one of the two polypeptides of the $\alpha 4$ integrin may be added to the reaction mixture. Control cells
25 expressing polypeptides other than the two polypeptides of the $\alpha 4$ integrin may be used, to distinguish between specific reactions between the first and second components and non-specific reactions between the $\alpha 4$ integrin and other surface proteins of the cells expressing the second component.

A product of the invention which disrupts an interaction causes the degree of
30 interaction to be reduced or substantially eliminated as compared to the degree of

interaction in the absence of the product. Preferred products cause a reduction in the degree of interaction as compared to that in the absence of the product of at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 99% or substantially
5 eliminate the interaction at a concentration of the product of 0.1mg ml^{-1} , 1mg ml^{-1} , 10mg ml^{-1} , 100mg ml^{-1} , 500mg ml^{-1} , 1mg ml^{-1} , 10mg ml^{-1} , 100mg ml^{-1} or 0.1g ml^{-1} . Any combination of the above percentage reductions in the degree of interaction and concentrations of product may be used to define a product of the invention, with greater disruption at lower concentrations being preferred. Preferred products of the
10 invention are those which show a 50% level of inhibition at a concentration of 1mg ml^{-1} or 0.5mg ml^{-1} .

The invention also provides a method for identifying a product which is capable of binding to amino acids 136 to 142 and/or amino acids 162 to 168 of OPN. The method comprises contacting a test product with a polypeptide which comprises
15 amino acids 136 to 142 amino acids of OPN or an analogous sequence thereto and/or amino acids 162 to 168 of OPN or an analogous sequence thereto. The ability of the test product to bind to the polypeptide is then determined.

In this type of assay the polypeptide may be the same as the first component described above for a two component assay. Any suitable assay format may be used.
20 Again, protocols which can be adapted for use in high through-put screens are preferred.

Any substance may be used as a test product. Suitable test products include combinatorial libraries, defined chemical entities, peptides and peptide mimetics, oligonucleotides and natural product libraries, such as display libraries (for example
25 phage display libraries). The test products may be used in an initial screen of, for example, ten products per reaction, and the products of batches which show antagonism tested individually. Furthermore, antibody products (for example, monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies, CDR-grafted antibodies and humanised antibodies) or fragments thereof may be
30 used.

It is preferable that products which disrupt the interaction between OPN and an $\alpha 4$ integrin do not disrupt the interaction between OPN and other integrins such as $\alpha v \beta 3$, $\alpha v \beta 5$, $\alpha v \beta 1$ and $\alpha 8 \beta 1$. That is, that products are specific for the interaction between OPN and an $\alpha 4$ integrin. It is possible that the new motifs represent motifs
5 unique to the interaction between OPN and $\alpha 4$ integrins and therefore that they represent targets for the specific disruption of the OPN/ $\alpha 4$ integrin interaction. This contrasts with the central RGD motif of OPN which determines its ability to bind to a number of integrins including $\alpha v \beta 3$, $\alpha v \beta 5$, $\alpha v \beta 1$ and $\alpha 8 \beta 1$.

Suitable products include functional derivatives of $\alpha 4$ integrins, fragments
10 derived from the OPN binding sites of an $\alpha 4$ integrin, mimetics of either an $\alpha 4$ integrin or a natural ligand of an $\alpha 4$ integrin, for example polypeptides based on OPN that mimic the structural region involved in $\alpha 4$ integrin-ligand binding interactions, polypeptides having a sequence corresponding to a functional binding domain of the natural ligand specific for an $\alpha 4$ integrin and antibodies which
15 immunoreact with either the motifs of OPN identified above or the regions of the $\alpha 4$ integrin responsible for those interactions.

Products of the invention may be in substantially purified form. They may be in substantially isolated form, in which case they will generally comprise at least 80% e.g. at least 90, 95, 97 or 99% by weight of the dry mass in the preparation. The
20 product is typically substantially free of other cellular components. The product may be used in such a substantially isolated, purified or free form in the method or be present in such forms in a kit.

Products of the invention may be used in a method of treatment of the human or animal body by therapy. In particular such substances may be used in immuno-
25 modulation or in the treatment of an inflammatory disease, granulomatous disease, fibrotic disease, multiple sclerosis or a bacterial or viral infection. Such substances may also be used for the manufacture of a medicament for use in immuno-modulation or in the treatment of an inflammatory disease, granulomatous disease, fibrotic disease, multiple sclerosis or a bacterial or viral infection. The condition of a

patient requiring an $\alpha 4$ integrin can be improved by administration of a product of the invention, typically the patient may require $\alpha 4\beta 1$. A therapeutically effective amount of a product of the invention may be given to a host in need thereof.

Preferably, the products used therapeutically will be those which can
5 modulate the interaction of an $\alpha 4$ integrin with the motif comprising amino acids 162 to 168 of OPN, or a functional variant thereof. The products may also be able to modulate the the interaction of an $\alpha 4$ integrin with the motif comprising amino acids 136 to 142 of OPN, or a functional variant thereof.

Examples of inflammatory diseases which may be treated with a product of
10 the invention include asthma, atherosclerosis, restenosis, ischaemia/reperfusion injury, arthritis, inflammatory bowel disease, type 1 diabetes, systemic lupus erythematosus or multiple organ dysfunction syndrome associated with trauma and sepsis. Examples of granulomatous disease which may be treated with a product of the invention include tuberculosis and sarcoidosis. An example of a bacterial infection is
15 tuberculosis. An example of a viral infection is influenza.

Products of the invention may be administered in a variety of dosage forms. Thus, they can be administered orally, for example as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules. The antagonists may also be administered parenterally, either subcutaneously, intravenously,
20 intramuscularly, intrasternally, transdermally or by infusion techniques. The inhibitors may also be administered as suppositories. A physician will be able to determine the required route of administration for each particular patient.

The formulation of a product of the invention will depend upon factors such as the nature of the exact antagonist, whether a pharmaceutical or veterinary use is
25 intended, etc. A product of the invention may be formulated for simultaneous, separate or sequential use.

A product of the invention is typically formulated for administration in the present invention with a pharmaceutically acceptable carrier or diluent. The pharmaceutical carrier or diluent may be, for example, an isotonic solution. For
30 example, solid oral forms may contain, together with the active compound, diluents,

e.g. lactose, dextrose, saccharose, cellulose, corn starch or potato starch; lubricants, e.g. silica, talc, stearic acid, magnesium or calcium stearate, and/or polyethylene glycols; binding agents; e.g. starches, gum arabic, gelatin, methylcellulose, carboxymethylcellulose or polyvinyl pyrrolidone; disaggregating agents, e.g. starch, 5 alginic acid, alginates or sodium starch glycolate; effervescing mixtures; dyestuffs; sweeteners; wetting agents, such as lecithin, polysorbates, laurylsulphates; and, in general, non-toxic and pharmacologically inactive substances used in pharmaceutical formulations. Such pharmaceutical preparations may be manufactured in known manner, for example, by means of mixing, granulating, tableting, sugar-coating, or 10 film-coating processes.

Liquid dispersions for oral administration may be syrups, emulsions or suspensions. The syrups may contain as carriers, for example, saccharose or saccharose with glycerine and/or mannitol and/or sorbitol.

Suspensions and emulsions may contain as carrier, for example a natural 15 gum, agar, sodium alginate, pectin, methylcellulose, carboxymethylcellulose, or polyvinyl alcohol. The suspensions or solutions for intramuscular injections may contain, together with the active compound, a pharmaceutically acceptable carrier, e.g. sterile water, olive oil, ethyl oleate, glycols, e.g. propylene glycol, and if desired, a suitable amount of lidocaine hydrochloride.

20 Solutions for intravenous administration or infusion may contain as carrier, for example, sterile water or preferably they may be in the form of sterile, aqueous, isotonic saline solutions.

A therapeutically effective amount of product of the invention is administered to a patient. The doses of a product of the invention may be determined according to 25 various parameters, especially according to the substance used; the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. Again, a physician will be able to determine the required route of administration and dosage for any particular patient. A typical daily dose is from about 0.1 to 50 mg per kg of body weight, according to the activity of the specific 30 inhibitor, the age, weight and conditions of the subject to be treated, the type and

severity of the degeneration and the frequency and route of administration.

Preferably, daily dosage levels are from 5 mg to 2 g.

The invention also provides a polypeptide which is (a) up to 50 amino acids in length and which comprises the amino acid sequence $X_2X_1\text{FPTDLP}X_3X_4$, or a
5 functional variant thereof and/or the amino acid sequence $X_7X_6X_5\text{SVVYGLR}$ or a functional variant thereof or (b) a fusion protein wherein the polypeptide in (a) is fused to a carrier polypeptide, wherein:

X_1 is absent or any amino acid and, if X_1 is present, X_2 is absent or any amino acid;

10 X_3 is absent or any amino acid and, if X_3 is present, X_4 is absent or any amino acid; and

X_5 is absent or any amino acid and, if X_5 is present, X_6 is absent or any amino acid and, if X_6 is present, X_7 is absent or any amino acid;

excluding the polypeptide consisting essentially of the sequence SVVYGLR:

15 Polypeptides (a) and (b), functional variants thereof and methods for producing such polypeptides and functional variants are described in detail above.

Polypeptides (a) and (b) and functional variants thereof may be used in a method for the identification of products which are capable of disrupting an interaction between amino acids 136 to 142 and/or amino acids 162 to 168 of human
20 OPN and an $\alpha 4$ integrin or in a method for the identification of a product which is capable of binding to amino acids 136 to 142 and/or amino acids 162 to 168 of human OPN and an $\alpha 4$ integrin. Typically, such methods will be a method as described above. Typically the $\alpha 4$ integrin will be $\alpha 4\beta 1$ or $\alpha 4\beta 7$.

Polypeptides (a) and (b) and functional variants thereof can also be used in a
25 method of treatment of the human or animal body by therapy. Thus, the said polypeptides may be used in the same way as products of the invention described above and formulated in an identical fashion for the treatment of the medical conditions described above. Cyclic polypeptides may be preferred for use in methods of treatment.

30 In addition, polypeptides (a) and (b) and functional variants thereof may be

used to disrupt the interaction between $\alpha 4$ integrin ligands other than OPN, including VCAM-1, the CS-1 fragment of fibronectin, MAdCAM CS-S, FN-1115, FN-III14 and the disintegrin EC3. In particular they may be used to disrupt the interaction of VCAM-1, the CS-1 fragment of fibronectin, CS-5, FNIII5, FNIII4, VCAM or EC3
5 with $\alpha 4\beta 1$ or the interaction of $\alpha 4\beta 7$ with VCAM, the CS-1 fragment of fibronectin, CS-5, FN-III5, FNIII4, and MAdCAM.

VCAM is involved in mediating exfiltration of cells from the blood stream and is expressed on the blood vessel wall. CS-1 is a fragment of fibronectin found, as an alternatively spliced region, in areas undergoing active or chronic
10 inflammation. This is found in the tissue rather than on the vessel wall and may influence cell behaviour once it has entered the inflamed area. Thus, polypeptide (a) and (b) and functional variants thereof may be used in methods of treatment of conditions in which VCAM, MAdCAM, CS-1, CS-5, FNIII5, FNIII14 and/or EC3 bind to an $\alpha 4$ integrin. Such conditions include those requiring immunomodulation
15 and inflammatory disease, granulomatous disease, fibrotic disease, multiple sclerosis or a bacterial or viral infection. The polypeptides may also be used for the manufacture of a medicament for use in immuno-modulation or in the treatment of an inflammatory disease, granulomatous disease, fibrotic disease, multiple sclerosis or a bacterial or viral infection.

20 Examples of inflammatory diseases which may be treated with a polypeptide (a) or (b) or a functional variant thereof, include asthma, atherosclerosis, restenosis, ischaemia/reperfusion injury, arthritis, inflammatory bowel disease, type 1 diabetes, systemic lupus erythematosus or multiple organ dysfunction syndrome associated with trauma and sepsis. Examples of granulomatous disease which may be treated
25 with a product of the invention include tuberculosis and sarcoidosis. An example of a bacterial infection is tuberculosis. An example of a viral infection is influenza.

Treatment or prevention of some disorders may require the targeting of a specific $\alpha 4$ integrins, for example of $\alpha 4\beta 1$ rather than of $\alpha 4\beta 7$ or vice versa. Accordingly, polypeptides, products or antibodies of the invention which affect the
30 activity of one of the $\alpha 4$ integrins but not the other may be used to treat such disorders.

Polypeptides (a) and (b) and functional variants thereof may be used in structural analysis methods for the design of products which are capable of disrupting the interaction between OPN and an $\alpha 4$ integrin and preferably specifically disrupt the interaction between one of the OPN motifs discussed above and an $\alpha 4$ integrin.

5 Various structural analysis methods for drug-design are known in the art, including, for example, molecular modelling, two-dimensional nuclear magnetic resonance (2-D NMR) analysis and x-ray crystallography.

In order to bind an $\alpha 4$ integrin the SVVYGLR motif, or functional variants of it, need a free acidic carboxyl terminus. This is normally generated *in vivo* by
10 cleavage at Arg 168 by thrombin to reveal such a group. The unusual way in which the binding site is generated also provides a target for therapeutic intervention.

Inhibitors which prevent the cleavage by thrombin may be used to prevent the generation of the carboxyl group at the acidic SVVYGLR binding site and hence be used to prevent interaction with $\alpha 4$ integrins and therefore to treat disorders
15 involving these integrins. Such inhibitors may prevent this cleavage in specific $\alpha 4$ integrin ligands rather than all of them and be used to treat disorders in which that specific ligand is involved.

The polypeptides of the invention which carry the SVVYGLR motif, or functional variant of it, may have a masking group which prevents the acid COOH
20 group of the Arginine interacting with an $\alpha 4$ integrin until the masking group is removed. The unmasking of such polypeptides may be carried out at the local site where the active polypeptide is required. This enables the modulators of activity of the invention $\alpha 4$ integrin to be activated at the specific site they are required.

Computer-based methods which permit the identification of compounds with
25 a desired molecular structure may be used to identify compounds whose structure is similar to all or a part of a compound of interest. Thus, compounds whose structure is similar to the polypeptides of the invention may be identified and such compounds may be capable of disrupting the interaction between OPN and an $\alpha 4$ integrin. Such computer-based methods fall into two-broad classes: database methods and *de novo*
30 design methods. In database methods the compound of interest is compared to all

compounds present in a database of chemical structures and compounds whose structure is in some way similar to the compound of interest are identified. The structures in the database are based either on experimental data, generated by NMR or x-ray crystallography, or modeled three-dimensional structures based on two-dimensional data. In *de novo* design methods, models of compounds whose structure is in some way similar to the compound of interest are generated by a computer program using information derived from known structures and/or theoretical rules.

The success of both database and *de novo* methods for identifying compounds with activities similar to the compound of interest depends on the identification of the functionally relevant portion of the compound of interest. For drugs, the functionally relevant portion is referred to as a pharmacophore. A pharmacophore is an arrangement of structural features and functional groups important for biological activity. Thus, pharmacophores may be identified which correspond to polypeptides of the invention. Such pharmacophores may allow the identification of products with a similar structure to polypeptides of the invention and which may be capable of interfering with the interaction between OPN and an $\alpha 4$ integrin.

Programs suitable for generating predicated three-dimensional structures from two-dimensional data include Concord (Tripos Associates, St. Louis, MO) and 3-D Builder (Chemical Design Ltd., Oxford, UK). Programs suitable for searching three-dimensional databases to identify molecules bearing a desired pharmacophore include MACCS-3D and ISIS/3D (Molecular Design Ltd., San Leandro, CA) and ChemDBS-3D (Chemical Design Ltd., Oxford, UK). Programs suitable for pharmacophore selection and design include DISCO (Abbott Laboratories, Abbott Park, IL) and catalyst (Bio-CAD Corp., Mountain view, CA). Databases of chemical structures are available from Cambridge Crystallographic Data Centre (Cambridge, UK) and Chemical Abstracts Service (Columbus, OH). *De novo* design programs include Ludi (Biosym Technologies Inc., San Diego, CA) and Aladdin (Daylight Chemical Information Systems, Irvine, CA). Such programs are well known to those skilled in the art.

The invention also provides monoclonal or polyclonal antibodies capable of

binding to a polypeptide (a) or (b) or a functional variant thereof. Typically, such antibodies will bind to a sequence comprising one or both of the motifs FPTDLPA or SVVYGLR or functional variants thereof and thus preferred antibodies of the invention are capable of disrupting an interaction between disrupting an interaction
5 between amino acids 136 to 142 and/or amino acids 162 to 168 of OPN and an $\alpha 4$ integrin.

Thus, an antibody of the invention may interact with an amino acid sequence which occurs within the region of OPN defined by amino acid 138 to 168 of SEQ ID NO: 1 or a sequence which is a functional variant thereof. The invention further
10 provides a process for the production of monoclonal or polyclonal antibodies of the invention.

Antibodies of the invention may be antibodies to human polypeptides or fragments thereof. Preferred antibodies are those which are able to discriminate between the two $\alpha 4$ integrin binding site in OPN. That is, preferred antibodies are
15 specific for one of the those binding sites.

For the purposes of this invention, the term "antibody", unless specified to the contrary, includes fragments of whole antibodies which maintain their binding activity for a polypeptide encoded by a polynucleotide of the invention, a polypeptide of the invention or a fragment thereof. Such fragments include Fv,
20 F(ab') and F(ab')₂ fragments, as well as single chain antibodies. Furthermore, the antibodies and fragment thereof may be chimeric antibodies, CDR-grafted antibodies or humanised antibodies.

Antibodies of the invention may be used, *inter alia*, in a method of treatment of the human or animal body by therapy. Antibodies may also be used in a method
25 for detecting polypeptides of the invention present in a biological sample, which method comprises:

- I providing an antibody of the invention;
- II incubating a biological sample with said antibody under conditions which allow for the formation of an antibody-antigen complex; and

III determining whether antibody-antigen complex comprising said antibody is formed.

A sample may be for example a tissue extract. Antibodies of the invention may be bound to a solid support and/or packaged into kits in a suitable container
5 along with suitable reagents, controls, instructions, etc.

The presence of osteopontin in a host may be indicative of a disease state. For example, elevated levels of osteopontin may be indicative of a condition requiring immuno-modulation, an inflammatory disease, granulomatous disease, fibrotic disease, multiple sclerosis or a bacterial or viral infection. Thus antibodies in
10 the invention may be useful in the diagnosis of such conditions. In a method for diagnosis of a condition linked to elevated OPN levels, a sample from a host is contacted with an antibody of the invention. Determining whether the antibody binds to polypeptides in the sample allows evaluation of the disease state of the host. Suitable samples include blood, serum and saliva. Alternatively, antibodies may be
15 linked to a revealing label and thus may be suitable for use in methods of *in vivo* OPN imaging.

Antibodies of the invention may also be used in a method of treatment of the human or animal body by therapy. They may be used and formulated as is described above for products of the invention. Humanized monoclonal antibodies are preferred
20 for use in methods of treatment and offer particular advantages over murine monoclonal antibodies. Specifically, humanized antibodies are not cleared from the circulation as rapidly as murine antibodies and are not as antigenic as murine antibodies.

Antibodies of the invention can be produced by any suitable method. Means
25 for preparing and characterising antibodies are well known in the art, see for example Harlow and Lane (1988) "Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. For example, an antibody may be produced by raising antibody in a host animal against the whole polypeptide or a fragment thereof, for example an antigenic epitope thereof, herein after the
30 "immunogen".

A method for producing a polyclonal antibody comprises immunising a suitable host animal, for example an experimental animal, with the immunogen and isolating immunoglobulins from the serum. The animal may therefore be inoculated with the immunogen, blood subsequently removed from the animal and the IgG
5 fraction purified.

A method for producing a monoclonal antibody comprises immortalising cells which produce the desired antibody. Hybridoma cells may be produced by fusing spleen cells from an inoculated experimental animal with tumour cells (Kohler and Milstein (1975) *Nature* 256, 495-497).

10 An immortalized cell producing the desired antibody may be selected by a conventional procedure. The hybridomas may be grown in culture or injected intraperitoneally for formation of ascites fluid or into the blood stream of an allogenic host or immunocompromised host. Human antibody may be prepared by *in vitro* immunisation of human lymphocytes, followed by transformation of the
15 lymphocytes with Epstein-Barr virus.

For the production of both monoclonal and polyclonal antibodies, the experimental animal is suitably a goat, rabbit, rat or mouse. If desired, the immunogen may be administered as a conjugate in which the immunogen is coupled, for example via a side chain of one of the amino acid residues, to a suitable carrier.
20 The carrier molecule is typically a physiologically acceptable carrier. The antibody obtained may be isolated and, if desired, purified.

Humanized antibodies may be obtained by replacing components of a non-human antibody with human components, without substantially interfering with the ability of the antibody to bind antigen.

25

The following Example illustrates the invention:

Example

Materials and Methods

Cell Culture

5 J6, HL60 and RPMI8866 cells were maintained in 1:1 RPMI 1640, Hepes modification (Gibco): Dulbecco's Minimum Essential Medium (DMEM), Hepes modification (Sigma) supplemented with glutamax (Gibco) and 10% fetal calf serum, FCS.

10 Antibodies and other reagents

Antibody clones used were as follows. The anti- β 1 integrin antibody clone 4B4 was obtained from Coulter. The anti- α 4 integrin clone HP2/1, anti- α 5 integrin clone SAM-1, anti- α L integrin clone 25.3, anti- α m integrin clone Bear1, anti- α v integrin clone AMF7, and anti- β 2 integrin clone 7E4 antibodies were obtained from
15 Immunotech. The anti- α 9 integrin clone Y9A2, anti- α v β 3 integrin clone LM609 and anti-b6 integrin clone 10D5 antibodies were obtained from Chemicon. The anti- β 7 function blocking antibody clone Fib504 was purified in house from a hybridoma obtained from the ATCC. The isotype control (MOPC21) was from Sigma. Phorbol 12-myristate 13-acetate (TPA) was obtained from Sigma. The α 4 β 1 inhibitor BIO1211
20 (Lin *et al*, J. Med. Chem. 42, 920-934, 1999) was synthesised in house. All oligonucleotides were obtained from GibcoBRL. Gels for polyacrylamide gel electrophoresis were obtained from Novex.

Flow Cytometry

25 For FACS analysis approximately 0.5×10^6 cells were used for each antibody. Cells were washed once in FACS buffer (PBS, 2.5% FCS, 0.1% sodium azide) and then incubated with 1 μ g of the relevant antibody for 10 minutes at room temperature. Cells were pelleted and washed two times in FACS buffer, before incubation for a further 10 minutes with anti-mouse IgG Alexa488-conjugated secondary antibody (Molecular

Probes). Finally cells were washed again twice in FACS buffer and resuspended in FACS buffer. Cells were analysed on a Coulter EPICS XL-1 flow cytometer.

Production of recombinant protein fragments

5 The full length human OPN cDNA (accession number X13694) was cloned by RT-PCR using the Superscript II kit (Stratagene) from kidney poly A+ RNA (Clontech). This cDNA was then used as a template to generate the fragments used in this study. All PCR reactions were performed using Pfu (Stratagene). The fragments encoding intact osteopontin lacking the leader sequence (aa17-314), the thrombin
10 cleaved fragment of osteopontin (aa17-168), a further 53 amino acid residue deletion (aa17-115), aa115-168 and 125-168 were generated by PCR. Each 5' primer incorporated a *Bam*HI site, while each 3' primer incorporated a terminal stop codon and a *Xba*I site.

 The resultant PCR products were first cloned into PCRscript, the sequence
15 verified, then subcloned as a *Bam*HI/*Xba*I fragment into a modified pGEX-2T vector (a gift from Christine Ellis, GlaxoWellcome). The pGEX-FN8-10 construct (encoding repeats 8-10 of fibronectin) was similarly generated by cloning a HindIII / SphI PCR generated fragment into the same modified pGEX-2T vector. The pGEX-CS-1 construct was obtained from Neil Burden (GlaxoWellcome, UK). The short overlapping peptides
20 spanning aa125-168 were constructed by annealing complementary oligos and ligating into the *Bam*HI/*Xba*I site of the modified pGEX-2T vector. Each pair of oligos were designed such that when annealed they mimicked a digested *Bam*HI site at the 5' end and a *Xba*I site at the 3' end. The 3' end of each pair of annealed oligos also included a terminal stop codon before the restriction site. The oligos used in this study are shown
25 in Table 1 below

Oligo Name	Sequence 5'→3'
STB10a – aa17 (5')	ACTCTGGATCCATACCAAGTTAAACAGGCTGATTCT
STB20 - aa314 (3')	AGTCTCTAGATTAATTGACCTCAGAAGATGCACTATC
STB32 - aa168 (3')	AGTCTCTAGATTACCTCAGTCCATAAACCACACTATC
ThrRAD – aa168RAD (3')	AGTCTCTAGATTACCTCAGTCCATAAACCACACTATCAGCTCG
STB30 - aa115 (3')	AGTCTCTAGATTAATCAGTGTCTATCTACATCATCAGA
STB long – aa115 (5')	AGTCGGATCCGATTCTCACCAGTCTGATGAG
STB short - aa125 (5')	AGTCGGATCCCATTCTGATGAATCTGATGA
STB54A – aa150-169 antisense	CTAGATTACCTCAGTCCATAAACCACACTATCAGCTCGGCCATCATATGTGTCTACA
STB54 - aa150-169 sense	GATCTGTAGACACATATGATGGCCGAGCTGATAGTGTGGTTTATGGACTGAGGTAA
STB53A – aa146-160 antisense	CTAGATTAAAGCTCGGCCATCATATGTGTCTACTGTGGGGACAACTGGAGTGAAA
STB53 - aa146-160 sense	GATCTTTCACTCCAGTTGTCCCCACAGTAGACACATATGATGGCCGAGCTTAAT
STB52A – aa139-153 antisense	CTAGATTATACTGTGGGGACAACTGGAGTGAAAACCTCGGTTGCTGGCAGGTCA
STB52 - aa139-153 sense	GATCTGACCTGCCAGCAACCGAAGTTTTCACTCCAGTTGTCCCCACAGTATAAT
STB51A – aa132-146 antisense	CTAGATTAGAAAACCTCGGTTGCTGGCAGGTCCGTGGGAAAATCAGTGACCAGA
STB51 - aa132-146 sense	GATCTCTGGTCACTGATTTTCCCACGGACCTGCCAGCAACCGAAGTTTTCTAAT
STB50A – aa125-139 antisense	CTAGATTAGTCCGTGGGAAAATCAGTGACCAGTTCATCAGATTCATCAGAATGA
STB50 - aa125-139 sense	GATCTCATTCTGATGAATCTGATGAACTGGTCACTGATTTTCCCACGGACTAAT

Table 1. Oligos used to generate the osteopontin fragments described. STB10a - aa17 (5'), STB20 - aa314 (3'), STB32 - aa168 (3'), ThrRAD - aa168RAD (3'), STB30 - aa115 (3'), STB short - aa125 (5') were used as PCR primers to the 5' and 3' ends as indicated. STB54A - aa150-169 antisense, STB54 - aa150-169 sense, STB53A - aa146-160 antisense, STB53 - aa146-160 sense, STB52A - aa139-153 antisense, STB52 - aa139-153 sense, STB51A - aa132-146 antisense, STB51 - aa132-146 sense, STB50A - aa125-139 antisense, STB50 - aa125-139 sense were annealed together as appropriate and inserted into the modified pGEX vector as described.

All the GST fusion proteins of OPN were expressed using the UT5600 bacterial strain (New England Biolabs). The deficiency of the periplasmic protease in UT5600 enabled production of intact OPN, which is otherwise cleaved at lysines aa170, 172 or 174 when produced in XL-1 Blue cells. The GST-FN8-10 and GST-CS-1 fusion proteins were both expressed in the XL-1 blue strain of bacteria. After transformation with the appropriate vectors, ampicillin resistant colonies were grown overnight in 2TY media containing 100µg/ml ampicillin at 37°C. The overnight culture were back diluted 1:40 and grown for a further 2-3 hrs at 37°C. Protein expression was induced with 0.5 mM isopropyl-1-thio-B-D-galactopyranoside (IPTG) for 2hr at 37°C. Cells were pelleted and resuspended in lysis buffer (25 mM Hepes (pH7.5), 150 mM NaCl, 2 mM DTT with Complete Protease Inhibitors (Boehringer)), then lysed by sonication. The supernatant was prepared by centrifugation at 10 000 rpm and the GST fusion proteins adsorbed onto reduced glutathione conjugated sepharose beads (Pharmacia). Protein-bound beads were washed 4 times with 25 column volumes of 25 mM Hepes (pH7.5), 150 mM NaCl, 2 mM DTT before elution with 5mM reduced glutathione (Sigma) in 25 mM Hepes (pH7.5), 150 mM NaCl, 2 mM DTT. Peak fractions were pooled, concentrated and then dialysed into PBS. Protein concentrations were determined using the Coomassie Plus Protein Assay Reagent (Pierce). The integrity and purity of each preparation was analysed both by SDS-PAGE and by mass spectrometry.

The recombinant human zz-VCAM and zz-MAdCAM, comprising the extracellular domain of each molecule coupled to the immunoglobulin binding domain of protein-A, were expressed in baculovirus and purified using an IgG coupled affinity column.

Peptides

The parent peptide SVVYGLR-COOH, scrambled peptide GRVLYSV-COOH and the capped peptide SVVYGLR-CONH₂ was synthesized in house. The synthetic peptides in which each residue of the SVVYGLR motif was individually mutated to Alanine were purchased from Cambridge Research Biochemicals Ltd.

Adhesion Assays

(i) General adhesion assays

5

All proteins were coated onto Maxisorp plates (Nunc) overnight at 4°C. Each protein was diluted in PBS at the concentrations indicated, and a total volume of 100µl added per well. Plates were washed twice in PBS then blocked with 3% BSA/PBS for 1hr at 37°C, and finally washed twice in PBS. Routinely HL60 cells were pelleted and washed once in HBSS (Sigma) then spread in 25mM Hepes 7.5, HBSS at $2 \times 10^6 \text{ ml}^{-1}$ (100µl per well) in the presence of 0.2 mM MnCl_2 and 50ng/ml TPA, with other additions as indicated. J6 cells were likewise pelleted and washed once in HBSS (Sigma) then spread in 25mM Hepes (pH7.5), HBSS at $2 \times 10^6 \text{ ml}^{-1}$ (100µl per well) in the presence of 0.2 mM MnCl_2 and other additions as indicated. For antibody inhibition cells were preincubated with the antibody (10µg/ml) on ice for 10 minutes. Cells were then allowed to attach for 35 minutes at 37°C, washed twice in PBS, once in ethanol, and fixed in ethanol for 20 minutes at room temperature. For quantitation cells were visualised by staining with 0.1% crystal violet (Sigma) for 10 minutes then lysed in 0.5% Triton X-100 (Sigma) and read at 570 nM in a Wallac Victor plate reader.

20

(ii) $\alpha 4 \beta 7$ adhesion assay

The recombinant zz-MAdCAM was absorbed onto Maxisorp plate pre-coated for 2.5 hours at 37°C with 100µL of human IgG, and then washed twice with PBS. For capture the recombinant zz-MAdCAM was diluted to the appropriate concentration in 3% BSA/PBS, 100µl added to each well, and incubated overnight at 4°C. Prior to use plates were washed twice in PBS.

For the $\alpha 4 \beta 7$ adhesion assay RPMI8866 cells were washed in cation free HBSS supplemented with 1mM EDTA, pelleted and labelled by incubating with BCECF (Molecular Probes) in cation-free HBSS at 37°C for 10 minutes. At this stage cells were

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also co-incubated with 1 μ l/ml human IgG to block endogenous Fc receptors. Cells were then washed once more in cation-free HBSS and then used in the adhesion assay at a final concentration of 3×10^6 cells ml^{-1} in the presence of the appropriate treatment as indicated. To prevent cells clumping the assay plate was finally centrifuged at 400 rpm for 3 minutes without the brake. Cells were then incubated for 20 minutes at 37°C and then washed twice in PBS before reading directly in a plate reader. For antibody inhibition studies the cells were preincubated with the appropriate antibody at 20 μ l/ml for 5 minutes at room temperature prior to being placed in the incubator.

Results

Expression of recombinant OPN fragments

To define the $\alpha 4\beta 1$ binding site within OPN we have used GST fusion proteins of human OPN as these have previously been shown to support adhesion as efficiently as the native protein. To obtain undegraded recombinant protein all fusions were expressed in the lon protease deficient strain, UT5600. Mass spectrometric analysis confirmed that the GST-OPN fusions were not degraded, and the proteins were greater than 95% pure as judged by SDS-PAGE (data not shown). A schematic representation of the larger fusion proteins used in this study is shown in Figure 1.

Integrin profiles of J6 and HL60 cells

To analyse the interaction between $\alpha 4\beta 1$ and OPN we have used two non-adherent cell lines, J6 and HL60. FACS analysis (Table 2) showed that these two cells types express a limited repertoire of integrins. Of the integrins analysed J6 express only $\alpha 5\beta 1$ and $\alpha 4\beta$, while HL60 express $\alpha 4\beta 1$, $\alpha 5\beta 1$ and $\alpha L\beta 2$. Studying the interaction of integrins with OPN is often complicated by the expression of a number of integrins capable of binding OPN on one cell type. This is particularly a problem when members of the αv integrin family are expressed as these integrins bind OPN with high affinity. The limited repertoire of integrin expression,

particularly the absence of αv integrins, makes J6 and HL60 cells ideal for studying the $\alpha 4 \beta 1$ interaction with OPN.

Cell Type	Integrin										
	IgG	$\alpha 1$	$\alpha v \beta 3$	$\beta 6$	$\beta 2$	$\alpha 5$	$\alpha 4$	αv	$\alpha 9$	αL	αm
HL60	1	31.1	1.4	3.6	17.8	19.1	19.8	3	0.9	19.6	1.2
J6	1	36.1	1.4	0.8	3.2	11.1	25.2	1.9	1	1.9	0.8

Table 2. FACS analysis of integrin expression on HL60 and J6 cells. The repertoire of integrins expressed on HL60 and J6 was analysed as indicated. Results are expressed as the median relative to the isotype control antibody.

HL60 and J6 cells bind intact osteopontin via $\alpha 4 \beta 1$

An interaction between $\alpha 4 \beta 1$ and bovine OPN was originally identified using HL60 cells, however binding required the presence of both TPA and a $\beta 1$ integrin activating antibody, 8A2, to promote $\alpha 4 \beta 1$ into a higher activation state. To assess the interaction with human OPN J6 and HL60 cells were allowed to adhere to a GST fusion protein corresponding to intact OPN (aa17-314) in the presence of function blocking antibodies (Figure 2). J6 cells adhered to intact OPN in the presence of Mn^{2+} alone (Figure 2A). To mimick the conditions required for HL60 binding [21], HL60 cells were allowed to adhere in the presence of both 50ng/ml TPA and Mn^{2+} , the Mn^{2+} substituting for the activating $\beta 1$ antibody 8A2 (Figure 2B). Adhesion of both cell types to intact OPN was inhibited by function blocking antibodies against both $\alpha 4$ and $\beta 1$ integrins. A function blocking antibody directed against $\alpha 5$ integrin reduced binding to OPN, but only to the same extent as an non-specific isotype control antibody. Therefore both J6 and HL60 cells adhere to full length OPN via $\alpha 4 \beta 1$, with little or no contribution from another adhesion receptor.

Mapping the $\alpha 4\beta 1$ binding site in OPN

To map the region of OPN that binds $\alpha 4\beta 1$, J6 cells were adhered to GST fusion proteins corresponding to aa17-314, 17-168 and 17-115 of OPN. aa17-168 represents the fragment of OPN generated *in vivo* by thrombin cleavage at aa168, while aa17-115 corresponds to the N-terminus of OPN truncated past the RGD motif, which interacts with the integrins, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 1$, $\alpha 8\beta 1$ and $\alpha 5\beta 1$. To ensure that binding to these fragments was mediated by $\alpha 4\beta 1$ cells were also adhered in the presence of the $\alpha 4\beta 1$ inhibitor BIO1211 (Lin *et al*, 1999). J6 cells adhered to intact (aa17-314) and thrombin cleaved (aa17-168) OPN, however making the additional truncation to aa115 (aa17-115) abrogated binding (Figure 3). As expected adhesion to aa17-314 was completely dependent upon $\alpha 4\beta 1$, being blocked by BIO1211. However adhesion to aa17-168 was not totally blocked by the $\alpha 4\beta 1$ inhibitor, although $\alpha 4\beta 1$ clearly bound this fragment. This additional adhesion to the thrombin cleaved fragment is mediated by $\alpha 5\beta 1$, which interacts with the RGD motif upon truncation of the protein to aa168. That BIO1211 specifically blocks $\alpha 4\beta 1$ mediated adhesion to CS-1 and not $\alpha 5\beta 1$ mediated adhesion to Fn repeats 8-10 demonstrates that it is specific for $\alpha 4\beta 1$ at the concentration used. Collectively this data localises the $\alpha 4\beta 1$ binding site to between aa115-168.

To confirm that this region (aa115-168) contains the $\alpha 4\beta 1$ binding site and to narrow the region down further, J6 cells were adhered to GST fusion proteins corresponding to aa115-168 and 125-168 of OPN, in the presence and absence of BIO1211 (Figure 4A). J6 cells bound both fusion protein via $\alpha 4\beta 1$, as adhesion was reduced by BIO1211. As both fusion proteins contain the RGD motif binding via $\alpha 5\beta 1$ is evident at higher matrix concentrations in the presence of BIO1211. $\alpha 4\beta 1$ can interact with a diverse range of motifs including RGD under certain conditions. To determine whether $\alpha 4\beta 1$ binds the RGD motif in OPN, the RGD in the thrombin cleaved fragment (aa17-168) was mutated to RAD (aa17-168RAD). This mutation ablates adhesion via RGD dependent integrins but did not affect binding of J6 cells to aa17-168RAD via $\alpha 4\beta 1$ (Figure 4B) Introducing this RAD mutation into the

thrombin cleaved fragment blocked adhesion via $\alpha 5 \beta 1$, as binding was completely blocked by BIO1211. The $\alpha 4 \beta 1$ binding site is therefore contained within aa125-168 and does not require a functional RGD motif.

To define the $\alpha 4 \beta 1$ binding site within aa125-168, the region was analysed in detail by expressing a series of overlapping GST fusion proteins (Figure 5A). To eliminate cell binding through the cryptic $\alpha 4 \beta 1$ site, the Gly of the RGD site was mutated to Ala in peptides 146-160 and 153-168. Surprisingly two fusion protein corresponding to aa132-146 and aa153-168 supported J6 cell adhesion (Figure 5B), which was mediated by $\alpha 4 \beta 1$ as BIO1211 blocked adhesion to both peptides (Figure 5C). The first sequence aa132-146 is non conserved, present only in human, bovine and possibly pig OPN, while the second sequence aa153-168 is conserved across all species (Fig. 6). This data is consistent with there being two $\alpha 4 \beta 1$ binding sites within the N-terminus of OPN capable of supporting J6 cell adhesion via $\alpha 4 \beta 1$.

The minimal sequences for the two sites are likely to be FPTDLPA (aa136-142) and RGDSVYGLR (aa159-168). The peptide FPTDLPA (aa136-142) lies within a non-conserved region, and would only be predicted to act as an $\alpha 4 \beta 1$ binding site in human, bovine and possibly pig OPN. However the second motif RGDSVYGLR (aa159-168) is conserved across all species (Figure 6). Although the data presented shows binding of J6 cells, experiments performed with HL60 cells were identical.

A synthetic peptide SVVYGLR but not FPTDLPA blocks binding to CS-1 by $\alpha 4 \beta 1$

The overlapping fusion proteins used to analyse $\alpha 4 \beta 1$ binding within the region aa125-168 suggest that the critical motifs in each fusion protein are FPTDLPA (within aa132-146) and RGDSVYGLR (within aa153-168). The site within aa153-168 can be narrowed further to SVVYGLR, as the RGD motif is not critical for binding. To determine whether the potential binding sites could act as competitive inhibitors of $\alpha 4 \beta 1$, two peptides, SVVYGLR and FPTDLPA, were synthesised and the ability of each to inhibit binding to a GST fusion protein corresponding to the CS-1 fragment of

fibronectin was assessed (Figure 7).

SVVYGLR, but not its scrambled control peptide blocked adhesion to CS-1 (Figure 7B). This peptide was only slightly less potent at blocking adhesion to CS-1 than the CS-1 peptide itself (Figure 7A), indicating that SVVYGLR is an effective
5 inhibitor of $\alpha 4\beta 1$ binding. Conversely the peptide FPTDLPA was unable to inhibit binding to CS-1 compared to the scrambled control at the concentration of CS-1 ligand (Figure 7C). It did however show weak inhibition of binding over the scrambled control peptide at lower matrix concentrations (data not shown), consistent with this peptide making a low affinity interaction with the integrin. This suggests that of the two
10 peptides identified, SVVYGLR is the major $\alpha 4\beta 1$ binding site within OPN, while FPTDLPA represents a second, lower affinity binding site.

The osteopontin SVVYGLR motif supports adhesion via $\alpha 4\beta 1$ and $\alpha 4\beta 7$

$\alpha 4\beta 1$ and $\alpha 4\beta 7$ are related integrins which recognise common sites in certain
15 ligands such as VCAM-1 and CS-1. These integrins, along with $\alpha 9\beta 1$, form a sub-family of receptors defined by both their sequence homology and overlapping ligand binding repertoire. To analyse the interaction of $\alpha 4\beta 7$ with the SVVYGLR motif RPMI8866 cells which express $\alpha 4\beta 7$, and small amounts of $\alpha 4\beta 1$ and $\alpha 5\beta 1$ were used. Importantly these cells do not express any detectable αv integrin one of the major
20 alternate sub-family of integrins known to interact with osteopontin.

To demonstrate that $\alpha 4\beta 7$ binds the SVVYGLR motif RPMI8866 cells were allowed to adhere to the GST fusion protein corresponding to amino acid residues 153-168 of osteopontin in the presence and absence of function-blocking anti-integrin antibodies (Figure 8). In the intact protein this sequence also contains the RGD motif
25 of osteopontin, which binds αv integrins and $\alpha 5\beta 1$, therefore to prevent interactions via the RGD motif the Gly was mutated to Ala. Adhesion of RPMI8866 cells to amino acid residues 153-168 was blocked by $\alpha 4$ and $\beta 7$ function blocking antibodies, but not by antibodies against $\beta 1$, αv or $\alpha 5$ integrin. This demonstrates that RPMI8866 cells bind the SVVYGLR motif of osteopontin via $\alpha 4\beta 7$. Although these cells express a small

amount of $\alpha 41$ this receptor is not making a significant contribution to binding as the $\beta 1$ anti-functional antibody had no effect.

A synthetic peptide SVVYGLR inhibits $\alpha 4\beta 7$ binding to MAdCAM

5 The peptide SVVYGLR inhibits $\alpha 4\beta 1$ binding to a GST fusion protein corresponding to the CS-1 alternatively spliced fragment of fibronectin (Figure 7). To confirm that this peptide could also act as an $\alpha 4\beta 7$ antagonist its ability to inhibit RPMI8866 cell adhesion to MAdCAM was assessed. RPMI8866 cells bind MAdCAM exclusively through $\alpha 4\beta 7$, as blocking antibodies against the $\alpha 4$ integrin subunit and the
10 $\beta 7$ integrin subunit but not the $\beta 1$ integrin subunit blocked adhesion to zz-MAdCAM (Figure 9). The SVVYGLR peptide inhibited adhesion of the RPMI8866 cells to MAdCAM, while the scrambled control peptide had no effect (Figure 10). This data demonstrates that the SVVYGLR motif derived from OPN also acts as an antagonist of $\alpha 4\beta 7$ as it blocks binding to MAdCAM.

15

Analysis of Structural elements of the SVVYGLR motif critical for inhibition of $\alpha 4$ integrins

As the SVVYGLR peptide effectively blocks adhesion of $\alpha 4\beta 1$ to CS-1 and $\alpha 4\beta 7$ to MAdCAM this approach was used to determine the structural elements of the
20 SVVYGLR motif that interact with $\alpha 4\beta 1$ and $\alpha 4\beta 7$. A number of the motifs known to interact with $\alpha 4$ integrins contain a critical acidic residue normally an Asp. One significant feature of the SVVYGLR motif is that it lacks an obvious acidic group, therefore understanding how this motif binds $\alpha 4$ integrins could have important implications for the design of small molecule antagonists.

25 The SVVYGLR site is located immediately N-terminal to the thrombin cleavage site, which occurs at Arg168. It is possible that thrombin cleavage at amino acid 168 reveals a free C-terminal acidic group (carboxylic acid) enabling the SVVYGLR motif to engage $\alpha 4$ integrins. To test this hypothesis a capped peptide SVVYGLR-CONH₂ was synthesised and tested for the ability to inhibit J6 and RPMI8866 cell adhesion

(Figure 11 A and B). Removing the acidic carboxy terminus abrogated the ability of this peptide to inhibit adhesion via both $\alpha 4\beta 1$ and $\alpha 4\beta 7$.

This data indicates that the free acidic carboxy terminus of the SVVYGLR motif is providing an acidic group which is essential for its interaction with $\alpha 4$ integrins.

5 Moreover this data confirms that thrombin cleavage of osteopontin at amino acid residue 168 is required for $\alpha 4\beta 1$ and $\alpha 4\beta 7$ ligation.

To further determine which other elements of the SVVYGLR motif are required for the interaction with $\alpha 4$ integrins, a series of synthetic peptides were generated in which each amino acid was individually mutated to alanine. The ability of each peptide
10 to block adhesion of J6 and RPMI8866 cells to GST-CS-1 and zz-MAdCAM respectively was then assayed. The ability of each peptide to inhibit binding is shown in Figure 12 expressed as a relative potency compared to the activity of the parent peptide SVVYGLR-COOH. This analysis clearly shows that Leu167 is also crucial.

Mutating this residue results in a relative potency similar to that observed with the
15 blocked peptide SVVYGLR-CONH₂ or the scrambled control (data not shown).

A number of other residues were also found to make a minor contribution to the pharmacophore in particular Val163 and Tyr165, as mutation of these reduced the potency against both integrins. The observation that Tyr165 makes only a minor contribution to the $\alpha 4$ pharmacophore is interesting as this residue appears to be critical
20 for the interaction with $\alpha 9\beta 1$. Surprisingly mutation of Arg168 has opposite effects on the potency of the peptide against $\alpha 4\beta 1$ and $\alpha 4\beta 7$. Mutating this residue to Ala results in a peptide that is more potent against $\alpha 4\beta 1$ but less potent against $\alpha 4\beta 7$. Therefore the constituent at this position may confer some selectivity between $\alpha 4\beta 1$ and $\alpha 4\beta 7$.

Collectively this data shows that Leu167 and the free carboxy terminus of Arg168 are
25 the most critical residues within the SVVYGLR motif for binding to $\alpha 4$ integrins. This data is summarised in Figure 13.

CLAIMS

1. A method for identifying a product which is capable of disrupting an interaction between amino acids 136 to 142 and/or amino acids 162 to 168 of human osteopontin (OPN) and an $\alpha 4$ integrin, which method comprises:

- 5 (i) providing, as a first component, a polypeptide which is (a) up to 50 amino acids in length and which comprises the amino acid sequence $X_2X_1\text{FPTDLP}X_3X_4$ or a functional variant thereof and/or the amino acid sequence $X_7X_6X_5\text{SVVYGLR}$ or a functional variant thereof or (b) a fusion protein wherein the polypeptide (a) is fused to a carrier
- 10 polypeptide, wherein:
- X_1 is absent or any amino acid and, if X_1 is present, X_2 is absent or any amino acid;
- X_3 is absent or any amino acid and, if X_3 is present, X_4 is absent or any amino acid; and
- 15 X_5 is absent or any amino acid and, if X_5 is present, X_6 is absent or any amino acid and, if X_6 is present, X_7 is absent or any amino acid;
- (ii) providing, as a second component, an $\alpha 4$ integrin or a functional variant thereof;
- (iii) contacting the two components with a test product under conditions
- 20 that would permit the two components to interact in the absence of the test product; and
- (iv) determining whether the test product is capable of disrupting the interaction between the first and second components.

2. A method for identifying a product which is capable of binding to

25 amino acids 136 to 142 and/or amino acids 162 to 168 of human OPN, which method comprises:

- (i) providing a first component as defined in step (i) of claim 1;
- (ii) contacting the first component with a test product under conditions
- 30 that, in the presence of an $\alpha 4$ integrin but the absence of the test product, would lead to an interaction between the first component and

an $\alpha 4$ integrin; and

- (iii) determining whether the test product is capable of binding to the first component.

3. A method according to claim 1 or 2, wherein X_1 is D and/or X_2 is T
5 and/or X_3 is T and/or X_4 is E.

4. A method according to claim 1 or 2, wherein X_5 is D and/or X_6 is G or
A and/or X_7 is R.

5. A method according to any one of the preceding claims wherein the
carrier polypeptide is glutathione-S-transferase.

10 6. A method according to any one of the preceding claims, wherein the
polypeptide in step (i) has the amino acid sequence consisting essentially of
FPTDLPA, DTFPTDLPA, FPTDLPATE or DTFPTDLPATE and/or SVVYGLR,
RGDSVVYGLR or RADSVVYGLR.

7. A test kit suitable for use in identifying a product which is capable of
15 disrupting an interaction between amino acids 136 to 142 and/or amino acids 162 to
168 of human OPN and an $\alpha 4$ integrin, which kit comprises:

- (a) a first component as defined in step (i) of claim 1; and
(b) an $\alpha 4$ integrin or a functional variant thereof.

8. A kit according to claim 7, further comprising:
20 (c) means for determining whether a test product disrupts the interaction
between the first component and the said $\alpha 4$ integrin or functional variant
thereof.

9. A test kit suitable for identifying a product which is capable of
binding to amino acids 136 to 142 and/or amino acids 162 to 168 of human OPN,
25 which kit comprises:

- (a) a first component as defined in step (i) of claim 1; and
(b) means for identifying whether a test product binds to the first
component.

10. A product identified by a method according to any one of the claims 1
30 to 6 or a test kit according to any one of claims 7 to 9.

11. A product according to claim 10 for use in a method of treatment of the human or animal body by therapy.

12. A product according to claim 11 for use in immuno-modulation or the treatment of an inflammatory disease, a granulomatous disease, fibrotic disease,
5 multiple sclerosis or a bacterial or viral infection.

13. A product according to claim 12 wherein the inflammatory disease is asthma, atherosclerosis, restenosis, ischaemia/reperfusion injury, arthritis, inflammatory bowel disease, type 1 diabetes, systemic lupus erythematosus or multiple organ dysfunction syndrome associated with trauma or sepsis.

10 14. A product according to claim 12, wherein the granulomatous disease is tuberculosis or sarcoidosis.

15. Use of a product as defined in claim 10 in the manufacture of a medicament for use in immuno-modulation or the treatment of an inflammatory disease, a granulomatous disease, fibrotic disease, multiple sclerosis or a bacterial or
15 viral infection.

16. Use according to claim 15, wherein the inflammatory disease is asthma, atherosclerosis, restenosis, ischaemia/reperfusion injury, arthritis, inflammatory bowel disease, type 1 diabetes, systemic lupus erythematosus or multiple organ dysfunction syndrome associated with trauma or sepsis.

20 17. Use according to claim 15, wherein the granulomatous disease is tuberculosis or sarcoidosis.

18. A pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent and, as active ingredient, a product according to claim 10.

25 19. A method for treating a host requiring immuno-modulation or suffering from an inflammatory disease, a granulomatous disease, fibrotic disease or multiple sclerosis which method comprises administering to the host an effective amount of a product as defined in claim 10.

30 20. A polypeptide (a) or (b) as defined in step (i) of claim 1, excluding the polypeptide SVVYGLR.

21. A polypeptide according to claim 20, wherein X_1 is D and/or X_2 is T and/or X_3 is T and/or X_4 is E.

22. A polypeptide according to claim 20, wherein X_5 is D and/or X_6 is G or A and/or X_7 is R.

5 23. A polypeptide according to any one of claims 20 to 22 consisting essentially of the amino acid sequence FPTDLPA, DTFPTDLPA, FPTDLPATE or DTFPTDLPATE and/or RGDSVVYGLR or RADSVVYGLR

24. An antibody capable of binding a polypeptide a polypeptide (a) or (b) as defined in step (i) of claim 1.

10 25. A polypeptide (a) or (b) as defined in step (i) of claim 1 or an antibody capable of binding a said polypeptide, for use in a method of treatment of the human or animal body by therapy.

26. A polypeptide or antibody according to claim 25 for use in immuno-modulation or the treatment of an inflammatory disease, a granulomatous disease,
15 fibrotic disease, multiple sclerosis or a bacterial or viral infection.

27. A polypeptide or antibody according to claim 26 wherein the inflammatory disease is asthma, atherosclerosis, restenosis, ischaemia/reperfusion injury, arthritis, inflammatory bowel disease, type 1 diabetes, systemic lupus erythematosus or multiple organ dysfunction syndrome associated with trauma or
20 sepsis.

28. A polypeptide or antibody according to claim 26, wherein the granulomatous disease is tuberculosis or sarcoidosis.

29. Use of a polypeptide (a) or (b) as defined in step (i) of claim 1 or an antibody capable of binding a said polypeptide, in the manufacture of a medicament
25 for use in immuno-modulation or the treatment of an inflammatory disease, a granulomatous disease, fibrotic disease, multiple sclerosis or a bacterial or viral infection.

30. Use according to claim 29, wherein the inflammatory disease is asthma, atherosclerosis, restenosis, ischaemia/reperfusion injury, arthritis,
30 inflammatory bowel disease, type 1 diabetes, systemic lupus erythematosus or

multiple organ dysfunction syndrome associated with trauma or sepsis.

31. Use according to claim 29, wherein the granulomatous disease is tuberculosis or sarcoidosis.

32. A pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent and, as active ingredient, a polypeptide (a) or (b) as defined in step (i) of claim 1 or an antibody capable of binding a said polypeptide.

33. A method for treating a host requiring immuno-modulation or suffering from an inflammatory disease, a granulomatous disease, fibrotic disease, multiple sclerosis or a bacterial or viral infection which method comprises administering to the host an effective amount of a polypeptide (a) or (b) as defined in step (i) of claim 1 or an antibody capable of binding a said polypeptide.

34. Use of a polypeptide (a) or (b) as defined in step (i) of claim 1 in a method for identifying a product which is capable of disrupting an interaction between amino acids 136 to 142 and/or amino acids 162 to 168 of human OPN and an $\alpha 4$ integrin.

35. Use of a polypeptide (a) or (b) as defined in step (i) of claim 1 in a method for identifying a product which is capable of binding to amino acids 136 to 142 and/or amino acids 162 to 168 of human OPN.

36. A method of diagnosis of a condition requiring immuno-modulation, an inflammatory disease, granulomatous disease, fibrotic disease, multiple sclerosis or a bacterial or viral infection, which method comprises contacting an antibody according to claim 24 with a biological sample from a human or animal subject and determining whether the antibody binds to amino acids 136 to 142 and/or amino acids 162 to 168 of human OPN, thereby to determine the disease state of the subject.

-1-

SEQUENCE LISTING

SEQUENCE LISTING

5 <110> GLAXO GROUP LIMITED

<120> MEDICAL PRODUCTS

10 <130> PG3848S

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1 5 10 15

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30 Tyr Asn Lys Tyr Pro Asp Ala Val Ala Thr Trp Leu Asn Pro Asp Pro
35 40 45

Ser Gln Lys Gln Asn Leu Leu Ala Pro Gln Asn Ala Val Ser Ser Glu
35 50 55 60

Glu Thr Asn Asp Phe Lys Gln Glu Thr Leu Pro Ser Lys Ser Asn Glu
65 70 75 80

40 Ser His Asp His Met Asp Asp Met Asp Asp Glu Asp Asp Asp Asp His
85 90 95

Val Asp Ser Gln Asp Ser Ile Asp Ser Asn Asp Ser Asp Asp Val Asp
100 105 110

45 Asp Thr Asp Asp Ser His Gln Ser Asp Glu Ser His His Ser Asp Glu
115 120 125

Ser Asp Glu Leu Val Thr Asp Phe Pro Thr Asp Leu Pro Ala Thr Glu
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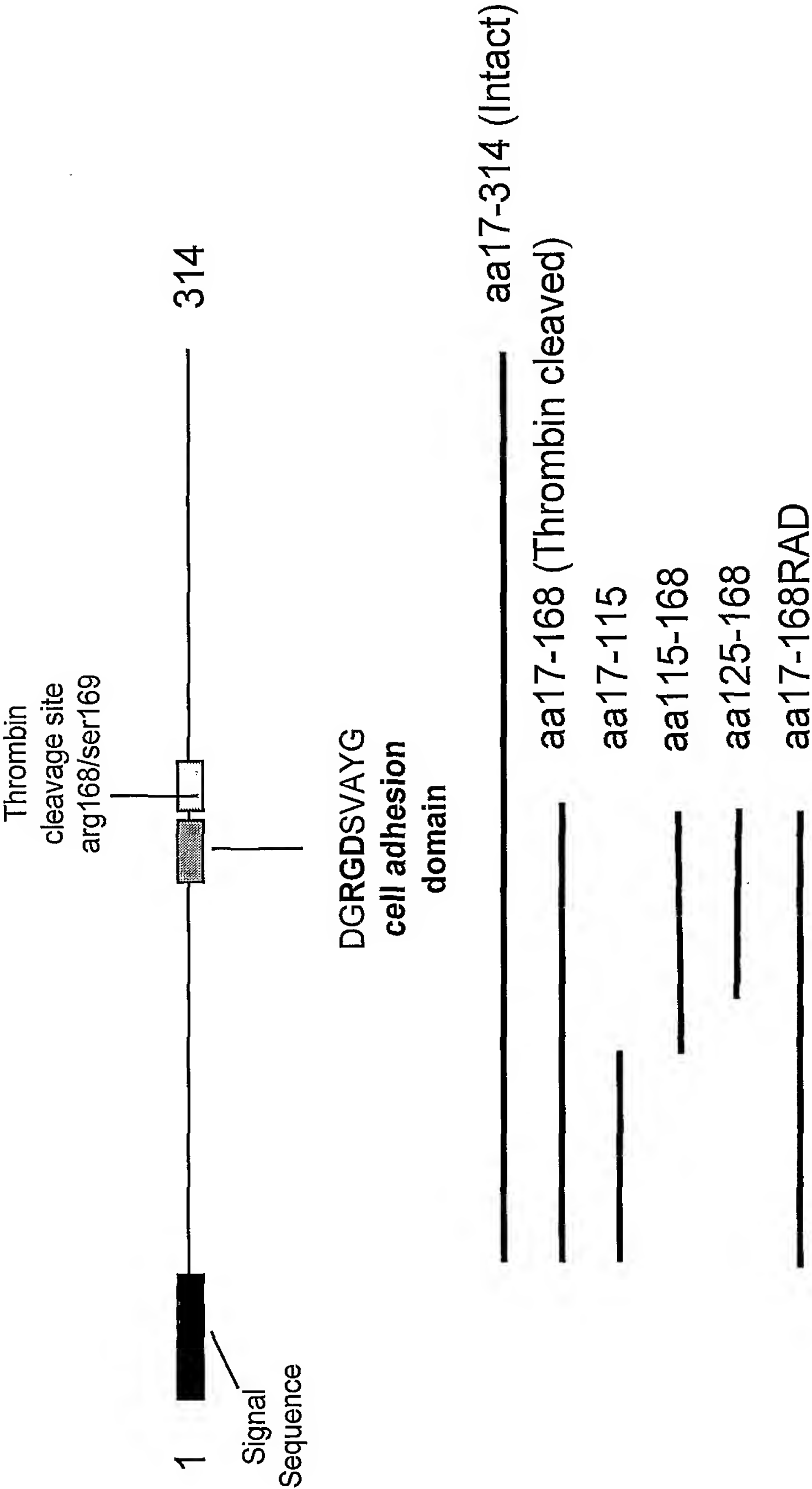


Figure 1

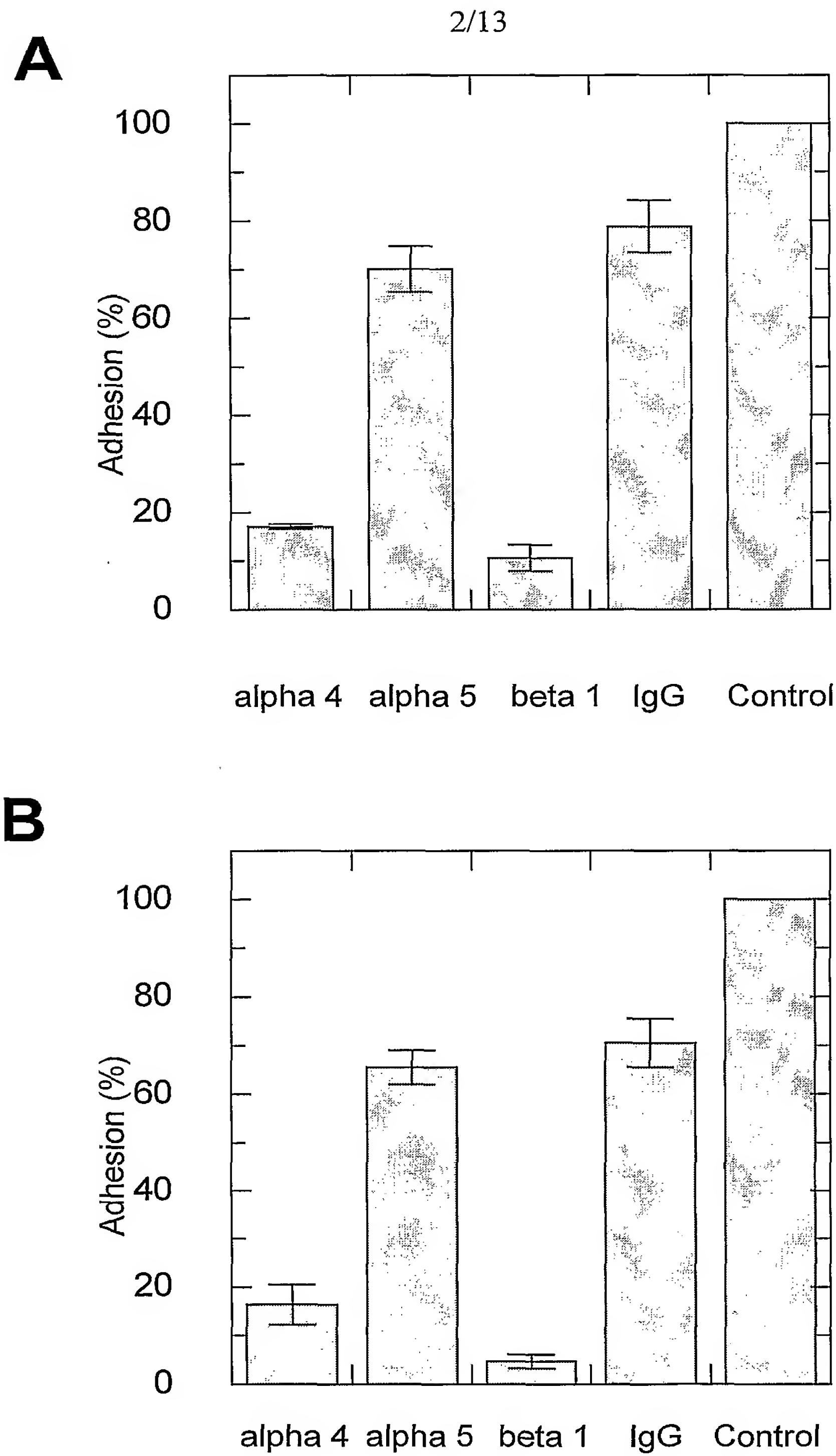


Figure 2

3/13

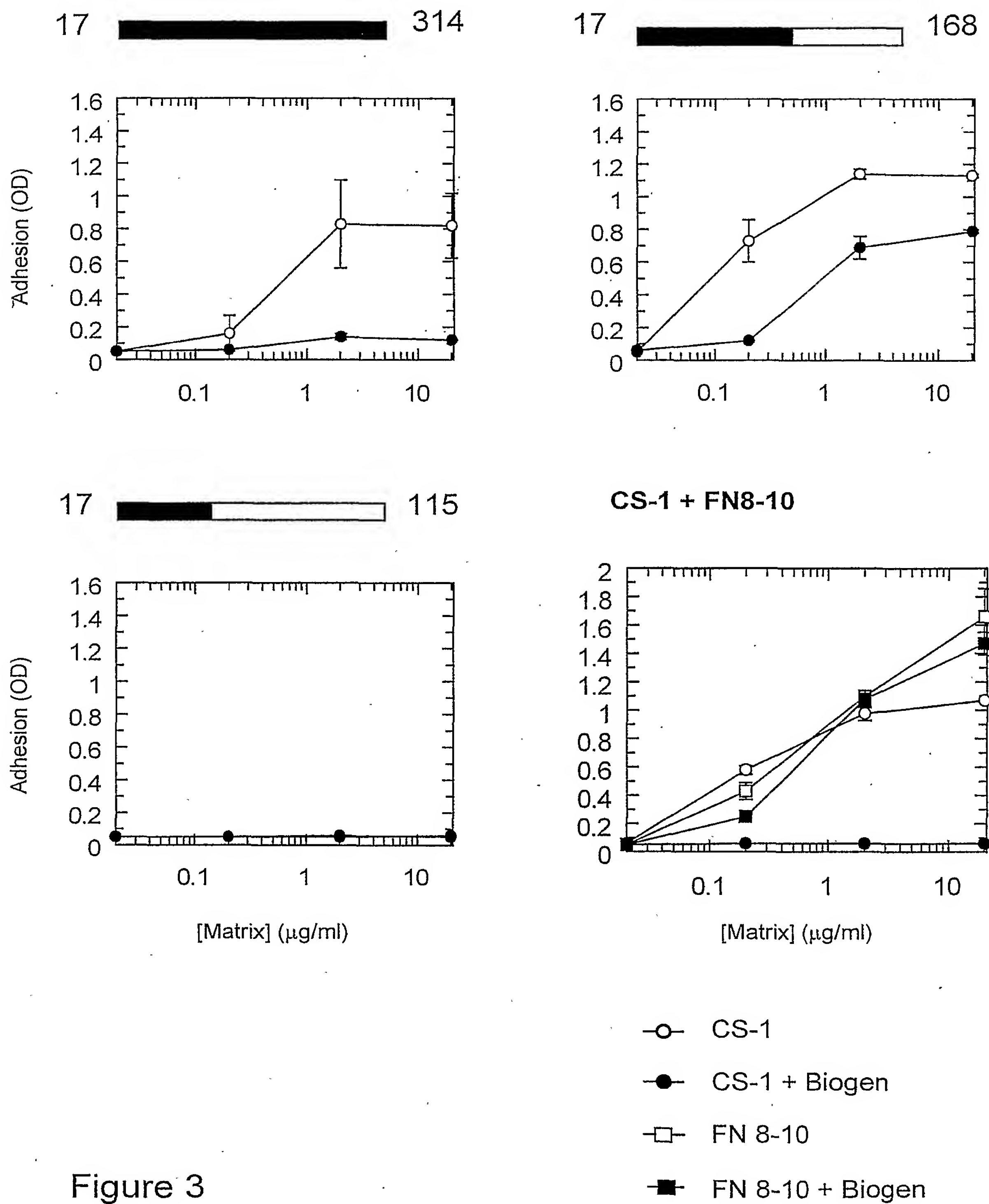


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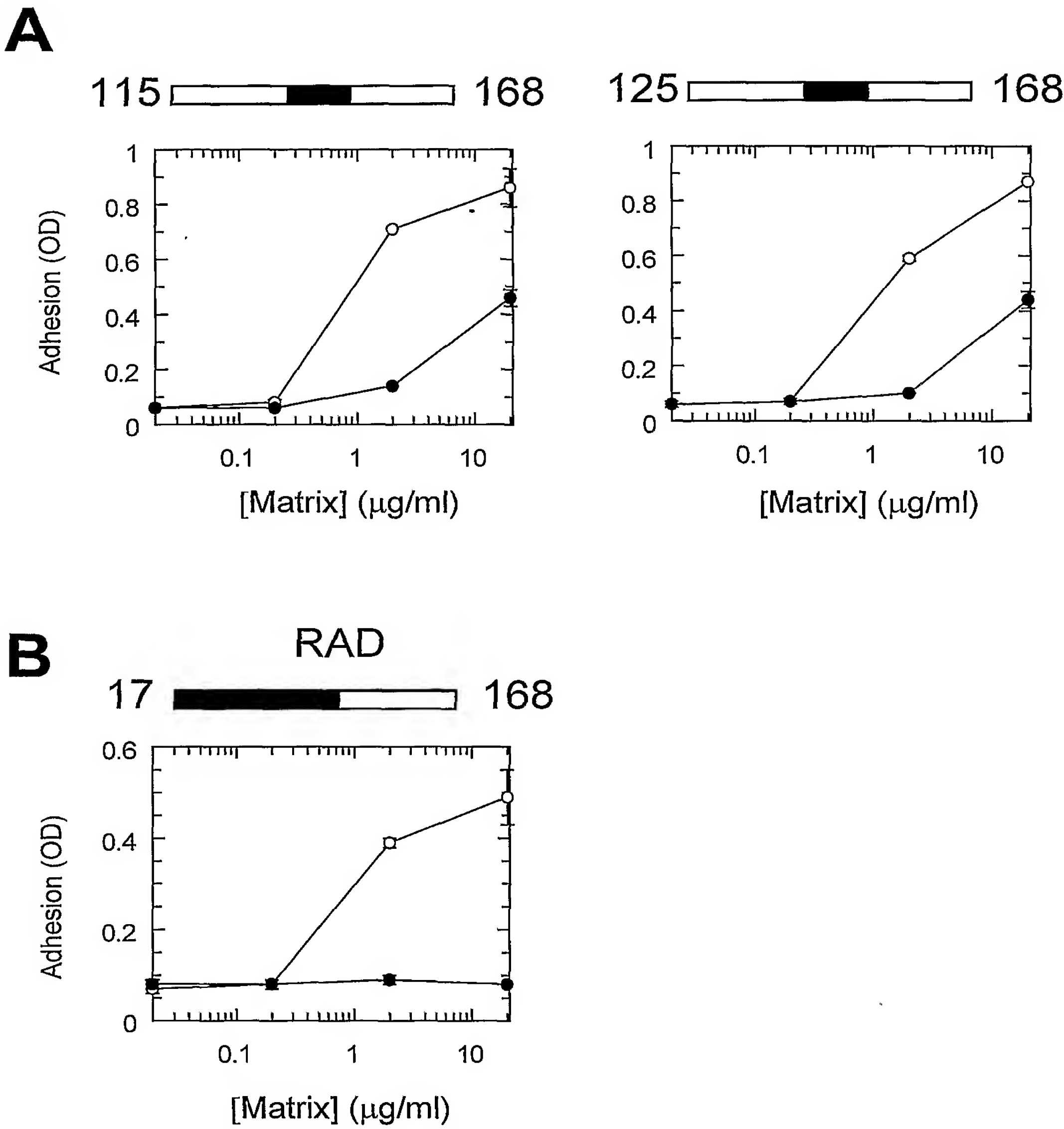
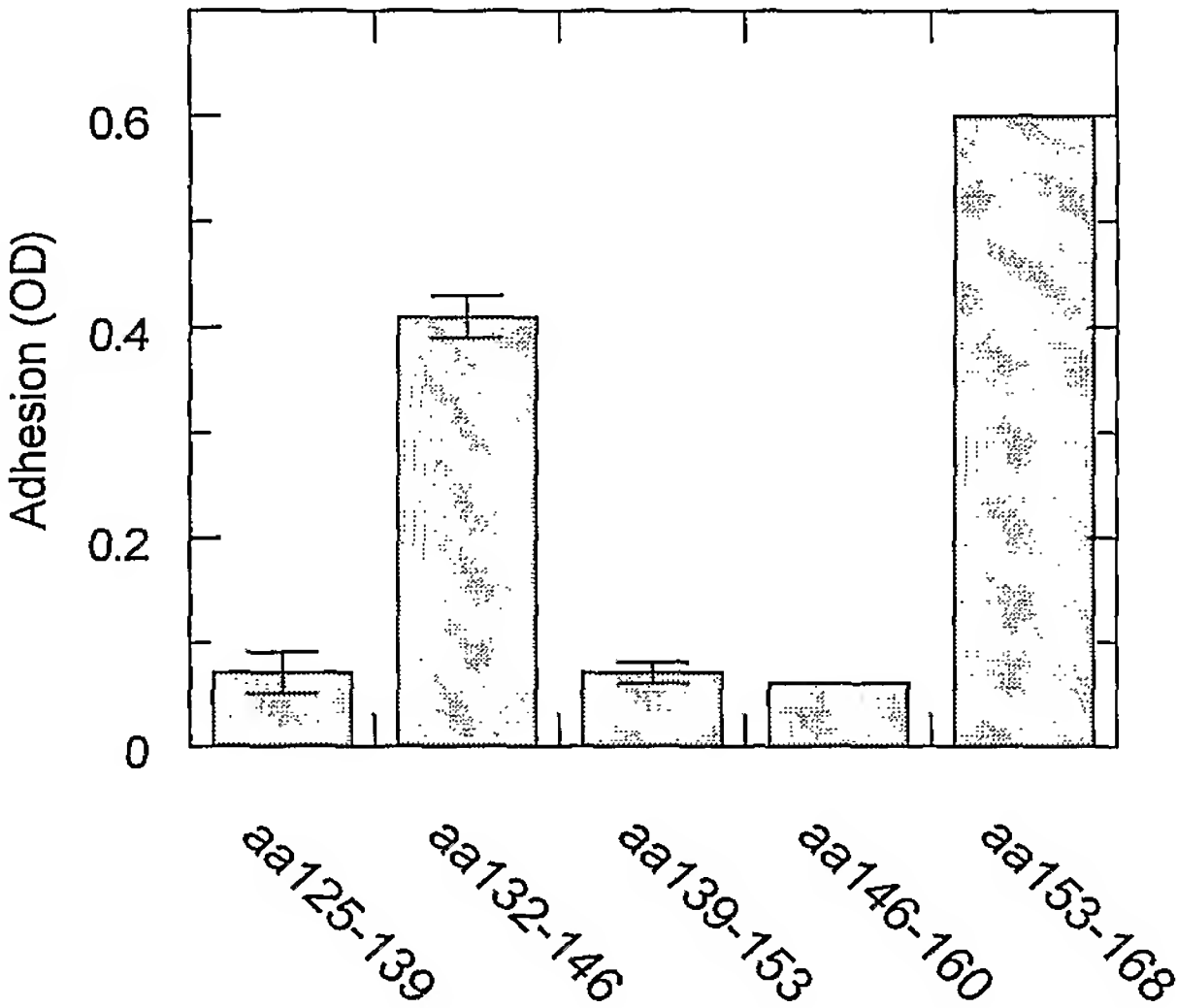


Figure 4

A

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132 LVTDFPTDLPATEVF 146
139 DLPATEVFTPVVPTV 153
146 FTPVVPTVDITYDGRA 160
153 VDTYDGRADSVVYGLR 168

B



C

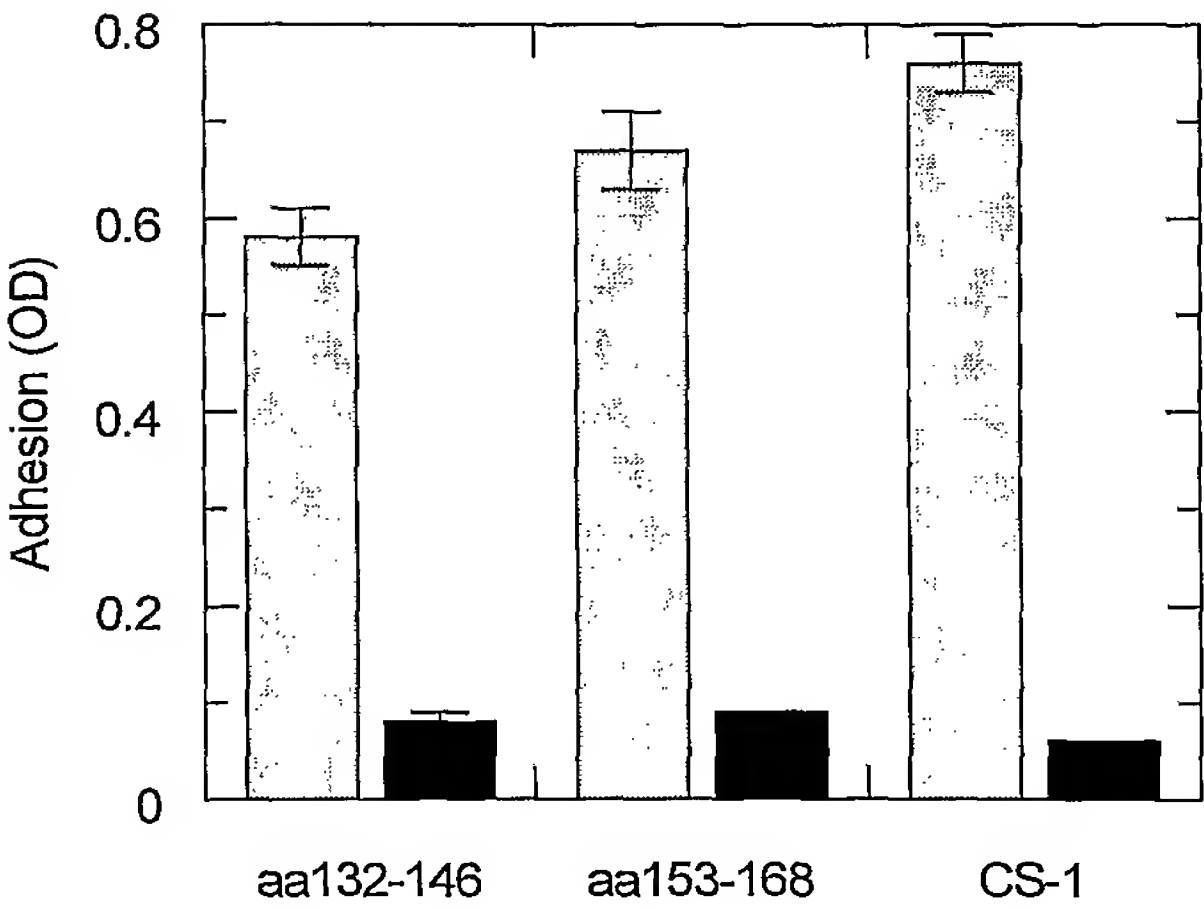


Figure 5

FIGURE 6

Human	.HSDESD ELVTDFPTDLPATEVF TPVPTVDTYDGRGDSVVYGLR
Bovine	.HSDESD--EVDFPTDIPTIAVFTFIPTESANDGRGDSVAYGLK
Pig	.HSDESD ELVTDFPTDTP -ATDVTPAVPTGDPNDGRGDSVVYGLR
Rabbit	HQSD ESD -EVTVPPTEDAA TTVFTEVVPTVF ETYDGRGDSVAYRLKR
Rat	.HSDESD---ESF-TASTQADVLTPIAPTVDVPDGRGDSLAYGLR
Mouse	.HSDESD---ETV-TASTQADTFTPIVPTVDVPNGRGDSLAYGLR

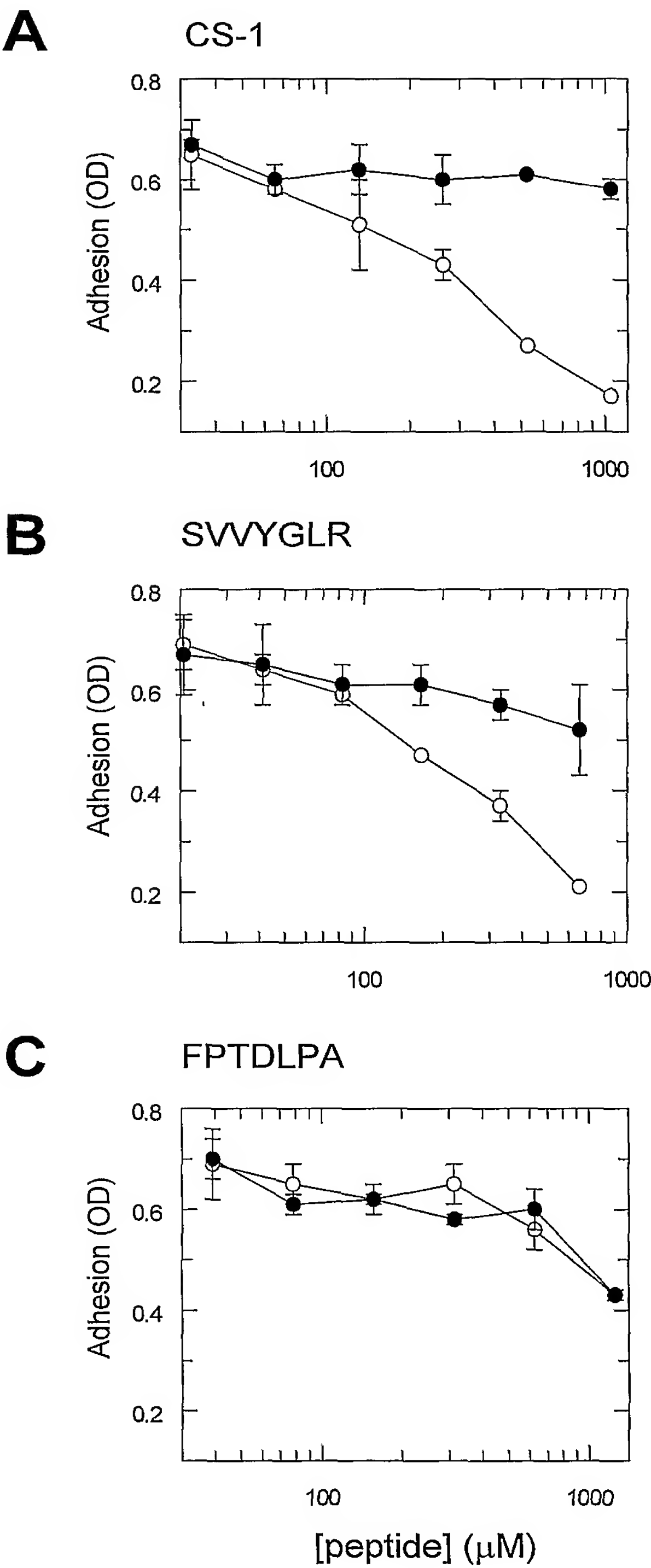
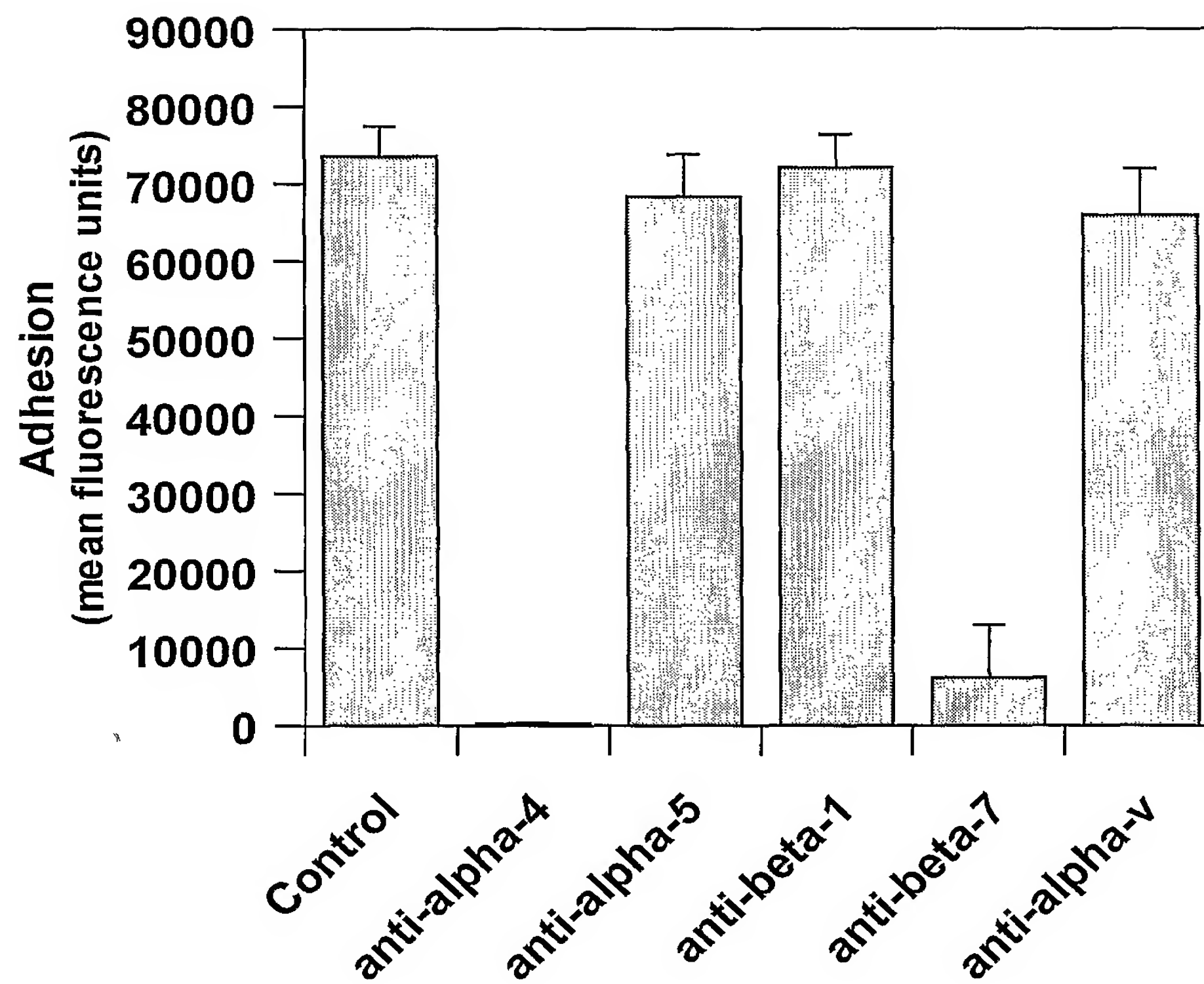


Figure 7

8/13

**Figure 8**

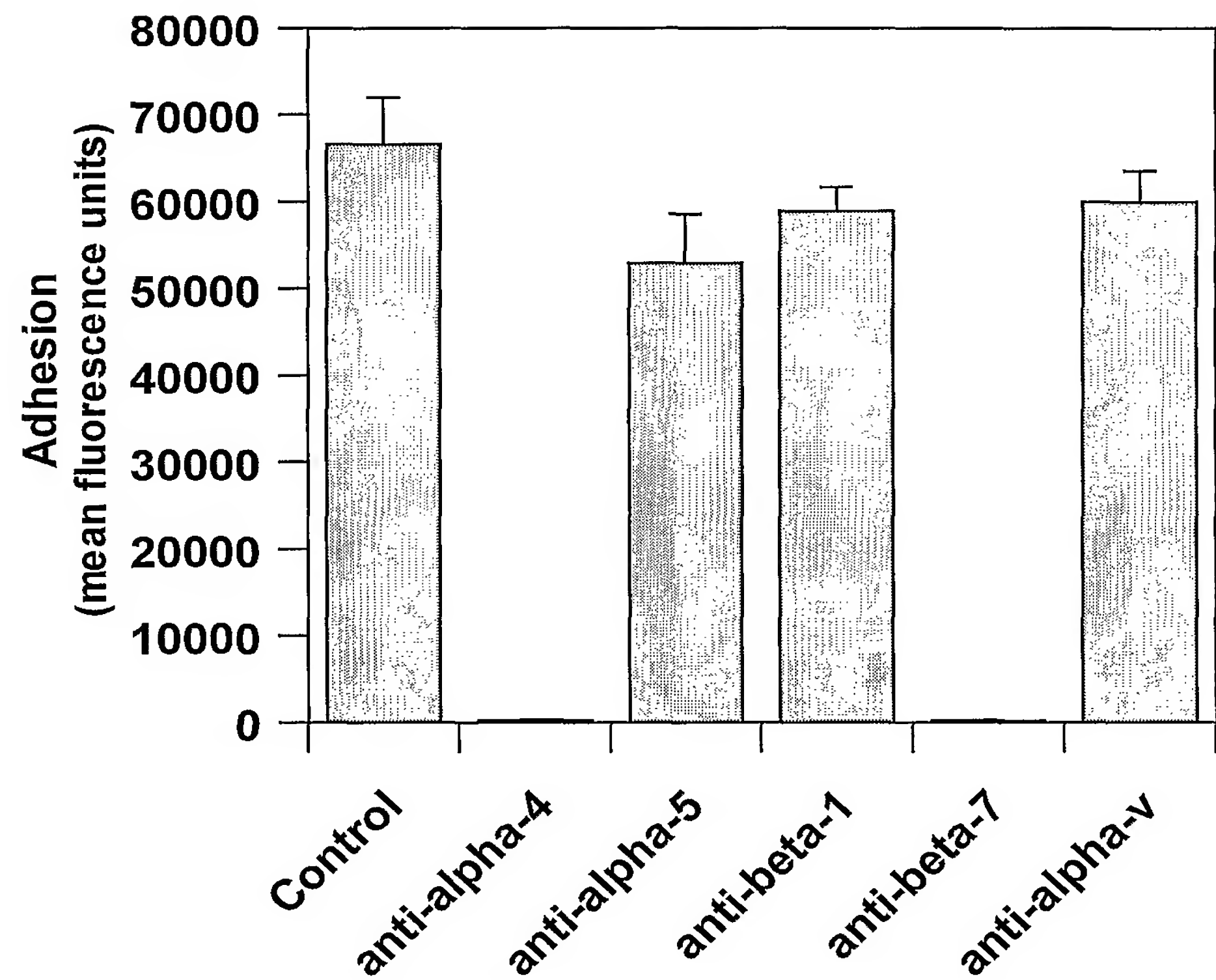
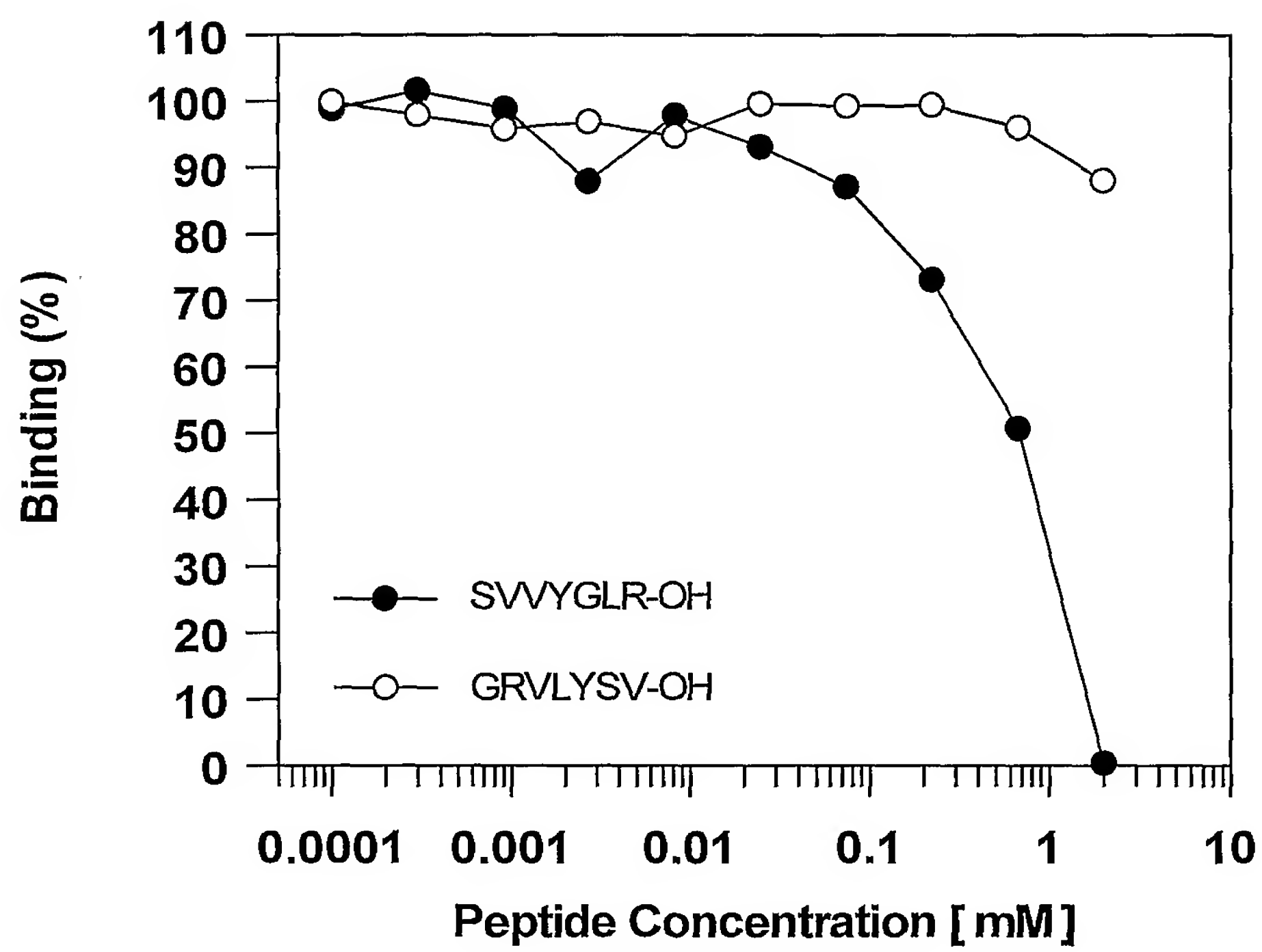


Figure 9

10/13

**Figure 10**

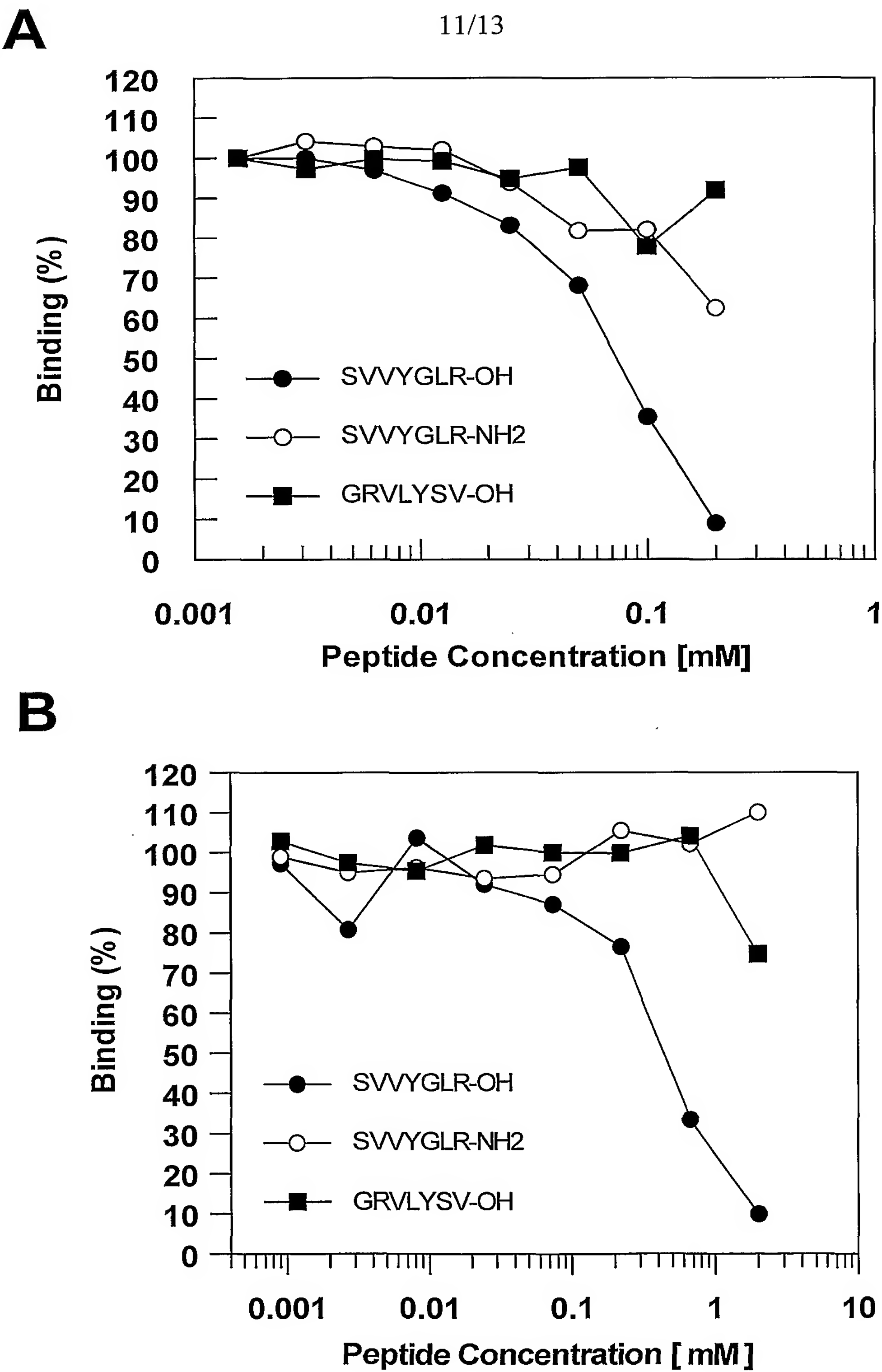


Figure 11

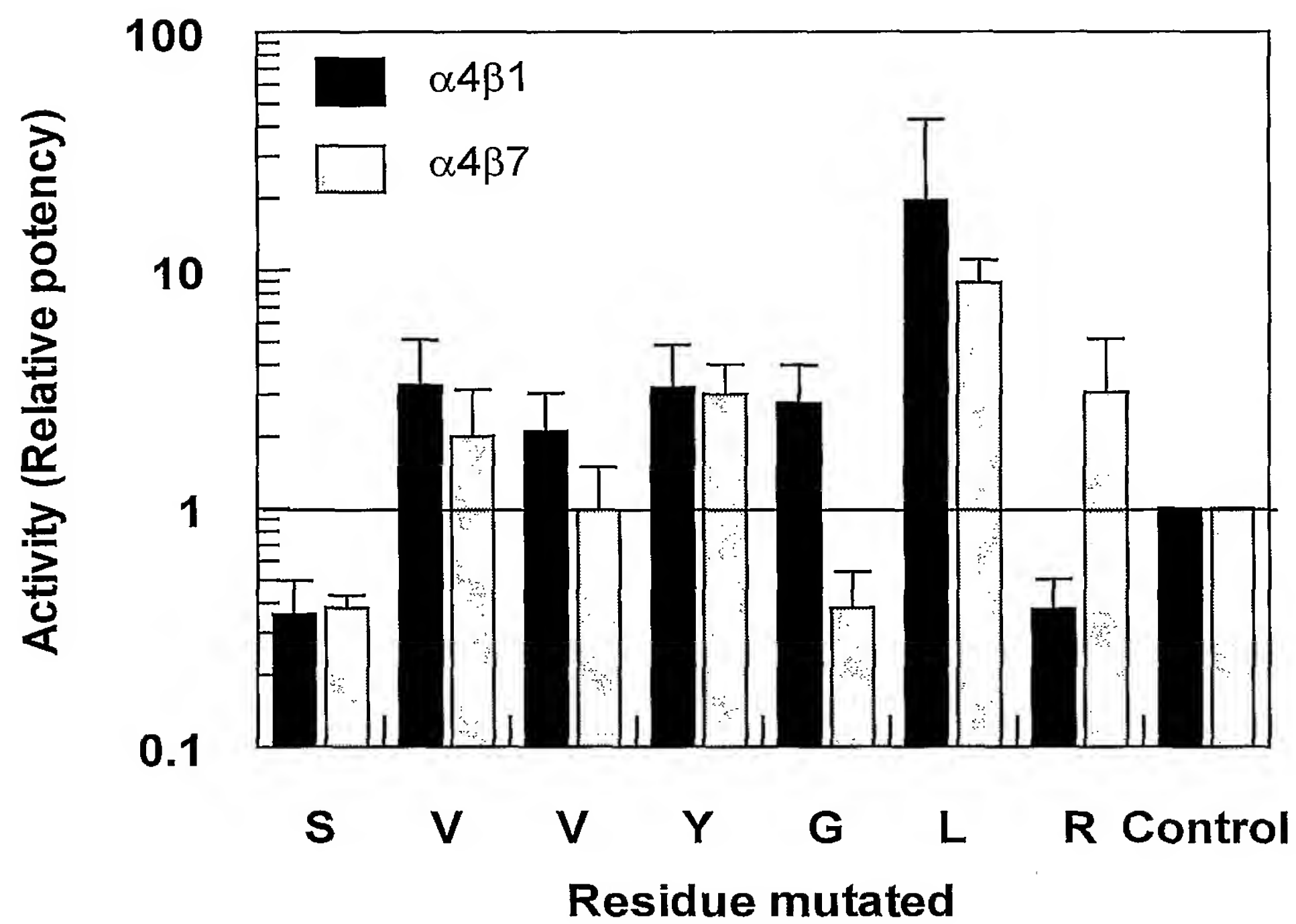
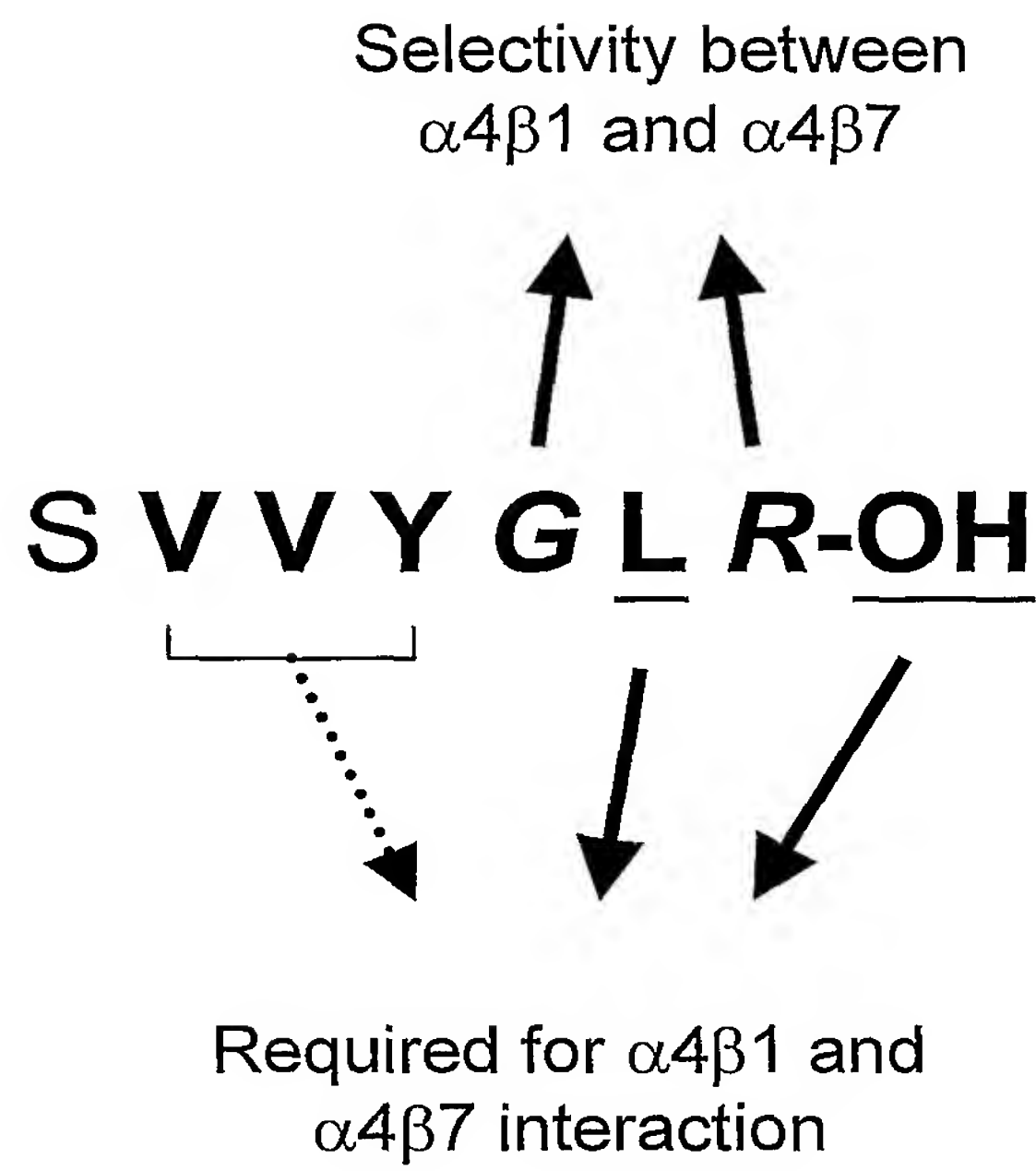


Figure 12

13/13

**Figure 13**

INTERNATIONAL SEARCH REPORT

Internat. Application No.
PCT/GB 01/01287

A. CLASSIFICATION OF SUBJECT MATTER IPC 7 G01N33/68 C07K14/52 C07K16/24 A61P29/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 G01N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, BIOSIS, WPI Data, PAJ, CHEM ABS Data, EMBASE, MEDLINE, EMBL		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BAYLESS KAYLA J ET AL: "Osteopontin is a ligand for the alpha4beta1 integrin." JOURNAL OF CELL SCIENCE, vol. 111, no. 9, 20 April 1998 (1998-04-20), pages 1165-1174, XP001020898 ISSN: 0021-9533 abstract page 1166, left-hand column, paragraph 2; figure 4 page 1171, right-hand column, paragraph 3 -page 1172, left-hand column, paragraph 1 ---	1-9
X	WO 99 08730 A (CHILDRENS MEDICAL CENTER) 25 February 1999 (1999-02-25) SEQ ID NO:6 --- -/--	20, 22, 23, 25
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
° Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
Date of the actual completion of the international search 22 August 2001		Date of mailing of the international search report 10/09/2001
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer Gundlach, B

INTERNATIONAL SEARCH REPORT

Intern: Application No
PCT/GB 01/01287

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with Indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 56405 A (GEN HOSPITAL CORP ;GUILFOILE PATRICK (US); WHITEHEAD BIOMEDICAL IN) 17 December 1998 (1998-12-17) abstract claims 10,11,14,18 ---	24
A	NASU K ET AL: "EXPRESSION OF WILD-TYPE AND MUTATED RABBIT OSTEOPONTIN IN ESCHERICHIA COLI, AND THEIR EFFECTS ON ADHESION AND MIGRATION OF P388D1 CELLS" BIOCHEMICAL JOURNAL, PORTLAND PRESS, LONDON, GB, vol. 307, no. 1, 1 April 1995 (1995-04-01), pages 257-265, XP002047130 ISSN: 0264-6021 abstract page 258, right-hand column, paragraph 5 figure 5 page 262, right-hand column, paragraph 2 page 264, left-hand column, paragraph 6 -right-hand column, paragraph 1 figures 5,6 ---	24
A	BARRY SIMON T ET AL: "A regulated interaction between alpha5beta1 integrin and osteopontin." BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS., vol. 267, no. 3, 27 January 2000 (2000-01-27), pages 764-769, XP001020783 ISSN: 0006-291X figure 1 ---	1,5,25
P,X	BARRY SIMON T ET AL: "Analysis of the alpha4beta1 integrin-osteopontin interaction." EXPERIMENTAL CELL RESEARCH, vol. 258, no. 2, 1 August 2000 (2000-08-01), pages 342-351, XP001020897 ISSN: 0014-4827 page 342, right-hand column, paragraph 3 -page 342, left-hand column, paragraph 1 page 343, left-hand column, paragraph 3 page 344, left-hand column, paragraph 2 -page 347, left-hand column, paragraph 2 --- -/--	20-23

INTERNATIONAL SEARCH REPORT

Intern application No
PCT/GB 01/01287

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>BAYLESS KAYLA J ET AL: "Identification of dual alpha4beta1 integrin binding sites within a 38 amino acid domain in the N-terminal thrombin fragment of human osteopontin." JOURNAL OF BIOLOGICAL CHEMISTRY, 'Online! no. 16, 25 January 2001 (2001-01-25), XP002175610 ISSN: 0021-9258 Retrieved from the Internet: <URL:http://www.jbc.org/cgi/content/abstract/M011392200v1> 'retrieved on 2001-08-22! abstract</p>	20-23
P,X	<p>--- MARCINKIEWICZ CESARY ET AL: "Inhibitory effects of MLDG-containing heterodimeric disintegrins reveal distinct structural requirements for interaction of the integrin alpha9beta1 with VCAM-1, tenascin-C, and osteopontin." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 275, no. 41, 13 October 2000 (2000-10-13), pages 31930-31937, XP001020781 ISSN: 0021-9258 abstract</p>	20,22,25
P,X	<p>--- WO 00 63236 A (ASHKAR SAMY ;CHILDRENS MEDICAL CENTER (US)) 26 October 2000 (2000-10-26) SEQ ID NO:7 claim 9</p>	20,22,25
T	<p>--- BAYLESS KAYLA J ET AL: "Identification of dual alpha4beta1 integrin binding sites within a 38 amino acid domain in the N-terminal thrombin fragment of human osteopontin." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 276, no. 16, 20 April 2001 (2001-04-20), pages 13483-13489, XP001020778 ISSN: 0021-9258 abstract</p> <p>-----</p>	

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claim 19 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

Continuation of Box I.2

Claims Nos.: 10-19

Present claims 10-19 relate to compounds, or the use of such compounds, or methods involving such compounds defined by reference to a desirable characteristic or property, namely being identifiable by the screening methods of claims 1-6.

The claims cover all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt compounds by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the screening methods per se and the compounds defined in claims 20-28.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internatl	lication No
PCT/GB	01/01287

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9908730 A	25-02-1999	AU 8783798 A EP 1003570 A	08-03-1999 31-05-2000
WO 9856405 A	17-12-1998	NONE	
WO 0063236 A	26-10-2000	AU 4356800 A	02-11-2000